ELECTRON MICROSCOPY OF THE PISCINE (*CARASSIUS AURATUS*) RENAL GLOMERULUS

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In a study of the comparative morphology of the vertebrate renal glomeruli, the avian (Pak Poy and Robertson, 1957), the marsupial (Pak Poy, 1957a) and the amphibian (Pak Poy, 1957b) glomeruli have been investigated with the electron microscope. The amphibian mesonephric glomerulus showed features which differed from the mammalian glomerulus, the structure of which has been previously described by numerous workers (Hall, 1953; Reid, 1954; Mueller *et al.*, 1955; Rinehart, 1955; Pease, 1955; Yamada, 1955). It was decided to study the mesonephric glomerulus of the common fresh water goldfish to see if the features of the mesonephric glomerulus of the frog were constant for other mesonephric glomeruli.

Materials and Methods.

The kidneys of young (approximately 6 months of age) fresh water goldfish (*Carassius auratus*) were prepared for electron microscopy in the same manner as described in previous papers (Pak Poy, 1957a, b) except that the tissue was fixed for one hour instead of two hours. Sections were examined in a Phillips type EM100 electron microscope.

RESULTS.

Glomerular loops. In sections of glomeruli the capillary loops are fewer than in mammals, but the lumen of an individual loop is much larger. Red cells with their nuclei showing varying stages of pyknosis and their cytoplasm varying degrees of haemoglobinisation are frequently present in the capillary lumen. In contrast with the findings in avian and marsupial glomeruli but in agreement with the findings in the amphibian glomerulus, the main bulk of endothelial cytoplasm with the nucleus is situated anywhere around the periphery of the lumen. The nucleus with its double nuclear membrane is generally large and ovoid and may be lobulated with a smooth surface, although occasionally the surface is obviously indented. A nucleolus may or may not be present. The main body of cytoplasm generally lies flat against the basement membrane and its periphery gives rise to the thin attenuated sheet of endothelial cytoplasm which lines the greater part of the capillary lumen. In one section, however,

the cell body lies free in the lumen and is connected to the thin endothelial sheet by a narrow stalk (Fig. 1). The cytoplasm contains small oval or round vesicles, some of which have a few granules attached, dense granules and a small number of mitochondria.

The thin endothelial sheet varies in thickness from 200A to 5000A and is perforated by "pores" measuring 300A to 1000A which are similar to those in other higher vertebrates. In places there are some interesting variations. In Fig. 2 three distinct processes of cytoplasm bound by definite cytoplasmic membranes lie step-like on one another giving rise to channels between the capillary lumen and the basement membrane. This arrangement may be merely the junction of five endothelial cells. In Fig. 15 an extremely thin layer of endothelial cytoplasm projects like an arc of a circle into the capillary lumen. The endothelial cytoplasmic membrane is usually sharply defined from the internal less dense layer of the capillary loop basement membrane although in some places this definition is absent (Fig. 19). Farquhar (1957), who does not describe an internal less dense layer of the capillary basement membrane suggests that the loss of definition between the endothelium and the dense layer indicates that the basement membrane is a product of the endothelium. However, the firm attachment of the lamina densa (see later) to the epithelial layer and the variable nature of the internal less dense layer suggests that the basement membrane is more closely related to the epithelial cell.

Epithelial cells. These cells are morphologically similar to those described in higher vertebrates but their pedicellar arrangement is not quite as complex. The terminal foot processes are extremely variable in size and shape. The small drop-like processes and the large layers of cytoplasm lying flat against the basement membrane, which were described in the amphibian mesonephric glomerulus are present in the piscine glomerulus. In some areas the filtration slit membrane (Yamada, 1955) is present between adjacent terminal foot processes. In addition to the typical epithelial cell lying free in Bowman's space there are present masses of extremely pale cytoplasm giving the appearance of "flowing" between the epithelial cells (Figs. 16 and 17). Scattered sparsely throughout this cytoplasm are oval vesicles of the endoplasmic reticulum and a small number of mitochondria. The cytoplasmic membrane is distinct in some areas, but is not visible in other areas where the cytoplasm merges imperceptibly with the material in Bowman's space. These masses of cytoplasm rest on Bowman's capsular epithelium and may contain nuclei, which appear morphologically similar to those of the visceral epithelial cells.

Basement membrane of capillary loop. In most sections the basement membrane is composed of the three layers, lamina rara externa, lamina densa, lamina rara interna, described in mammals by Pease (1955), Yamada (1955), Rhodin (1955), Bergstrand (1957) and Sagaguchi (1955), and in the bird by Pak Poy and Robertson (1957), and in the marsupials by Pak Poy (1957). The thickness

of the three layers together is approximately 950A in its most uniform areas, which is thinner than in mammals and birds. In places, however, there is marked thickening of the lamina rara interna with material which sometimes has the same appearance as serum (Figs. 3 and 6). In these regions the lamina densa remains adherent to the lamina rara externa and these two layers remain reasonably constant in measurement and relationship. This appearance is very similar to the sub-epithelial basement membrane described in the amphibian renal glomerulus (Pak Poy, 1957b). At the junction with "intercapillary tissue" in the plane of sectioning the lamina rara externa and lamina densa continue around the intercapillary tissue to the next capillary loop. The lamina densa may send off a smaller branch into the intercellular space of the "intercapillary tissue" at the junction of the capillary loop and "intercapillary tissue" or anywhere along the surface of the "intercapillary tissue".

"Intercapillary tissue". In the plane of sectioning, "intercapillary tissue", although it exists, is not as frequently seen as in the amphibian, avian and marsupial renal glomerulus. The wide intercellular spaces are filled with a less dense material with streaks of dense material running haphazardly through it. No collagen banding can be made out in it.

Pericapillary tissue. The lamina rara interna at the periphery of the capillary loop is sometimes extremely wide, pushing the attenuated endothelial sheet into the lumen. The space so created contains material which has either the same appearance as the serum in the capillary lumen or else a slightly less homogenous appearance (Figs. 3, 4 and 6). The lamina densa and lamina rara externa maintain their relationship with the epithelial pedicels and do not alter their dimensions appreciably, although the lamina densa may become a little thinner. Occasionally the lamina densa appears to split and gives rise to an indistinct dense layer beneath the endothelium. In this space between the endothelium and the sub-epithelial lamina densa, cells and projections of cytoplasm may be present (Fig. 11). These masses of cytoplasm have oval and longitudinal vesicles in the endoplasmic reticulum, dense granules and mitochondria but no features distinguishing them from endothelial cytoplasm except their position. The cells are separated from the endothelium by less dense intercellular material. This arrangement of pericapillary tissue is very similar to that found in the amphibian mesonephric glomerulus.

Bowman's capsule. There are two distinct types of cells present. Firstly, there is a type in which the nucleus and cytoplasm have the same electron density as the epithelial cells and which has been described in higher vertebrates. The nucleus is surrounded by a double nuclear membrane and is composed of collections of dense granules and filaments amongst which there may be present a nucleolus. The endoplasmic reticulum consists of oval or longitudinal vesicles some of which have attached granules. Dense granules and a few mitochondria in which the cristae may not be well formed occupy the rest of the cytoplasm.

Fig. 1. The electron micrograph shows an endothelial cell (E) lying free in the capillary lumen (C), with a narrow stalk-like attachment to the attenuated endothelial sheet (ES). A nucleated red cell (RBC) is also present in the lumen. Epithelial cells (H) are present in Bowman's space and epithelial pedicels (P) are projecting onto the capillary wall. × 23,000.

Fig. 2. The electron micrograph shows projections of endothelial cytoplasm (ES) lying step-like on one another. Their junctions may represent tangential sectioning through endothelial "pores" or boundary lines between endothelial cells. A poorly haemoglobinised red cell (RC) is present in the capillary lumen. Pedicels (P) rest on the capillary basement membrane. × 40,000.

Fig. 3. The electron micrograph shows a very much widened lamina rara interna which has been designated pericapillary space (PS) in mesonephric glomeruli of fish and amphibia. Endothelial sheet (ES) is one boundary and the other is the lamina densa (L) of the basement membrane. An epithelial cell (H) shows trabeculae and pedicel (P). A red cell (RC) is present in the capillary lumen (C). × 30,000.

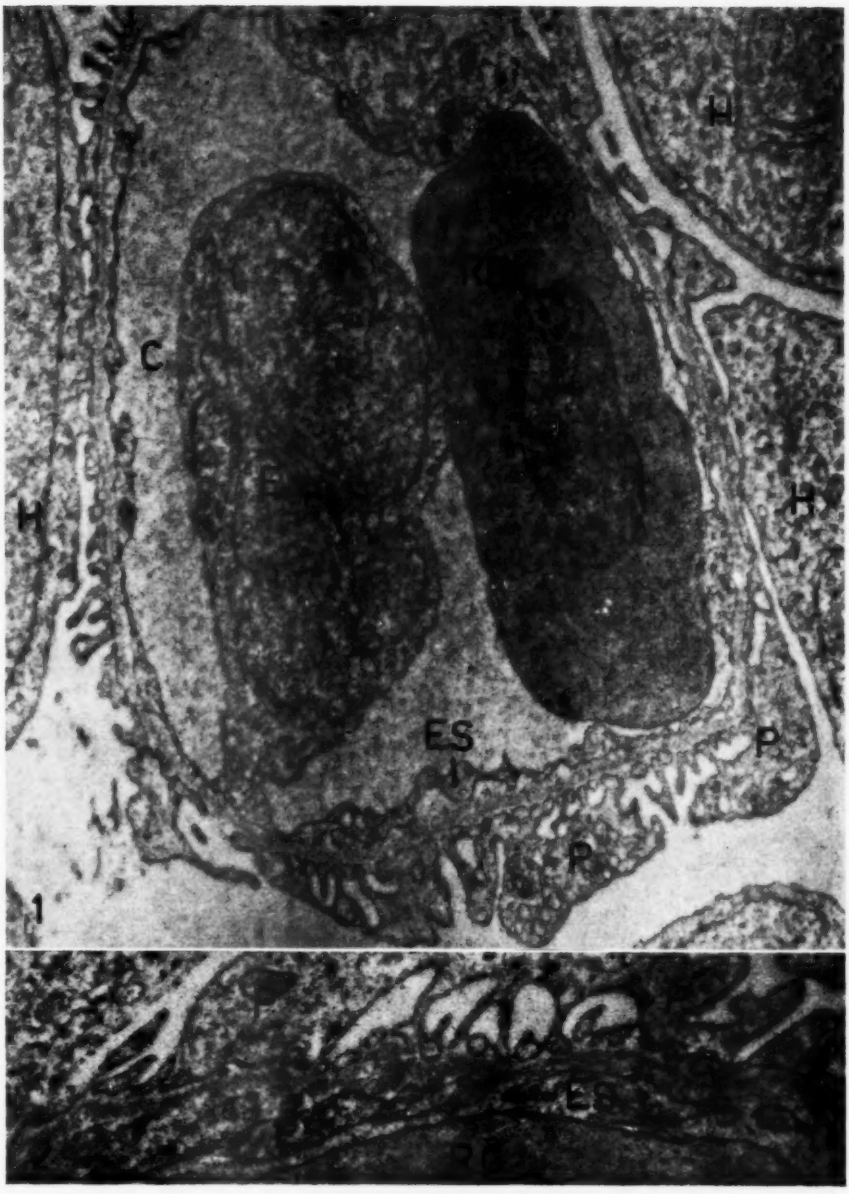
Fig. 4. The electron micrograph shows the gradual widening of the lamina rara interna into a small pericapillary space (V). It is apparent that the lamina densa (L) of the capillary basement membrane adheres to the epithelial pedicels (P). An epithelial cell with nuclei is at (H). A nucleated red cell (RC) is present in the capillary lumen (C) which is lined by the thin attenuated endothelial sheet (ES). × 30,000.

Fig. 5. The electron micrograph shows a flattened Bowman's capsular cell with its nucleus at (N) and cytoplasm (G). The cytoplasm has relatively the same density as epithelial cell cytoplasm. On the right a junction of two cells is seen. Bowman's capsular basement membrane is represented by a thin dense layer (K). × 22,000.

Fig. 6. The electron micrograph shows a pericapillary space (V) filled with material which has relatively the same electron density as the material in the capillary lumen (C) which contains a red cell (RC). The arrow indicates a "pore" or more probably an artefactual tear in the endothelial sheet. Pedicels are indicated at (P). × 30,000.

Fig. 7. The electron micrograph shows "pores" indicated by arrows measuring 200Å to 1000Å in the endothelial lining of the capillary lumen (C). Pedicels (P) rest on the basement membrane. × 45,000.

Fig. 8. The electron micrograph shows drop-like pedicels (D) on the basement membrane of a capillary lumen (C). The epithelial cell (H) can be seen to give rise to one of these drop-like pedicels. × 40,000.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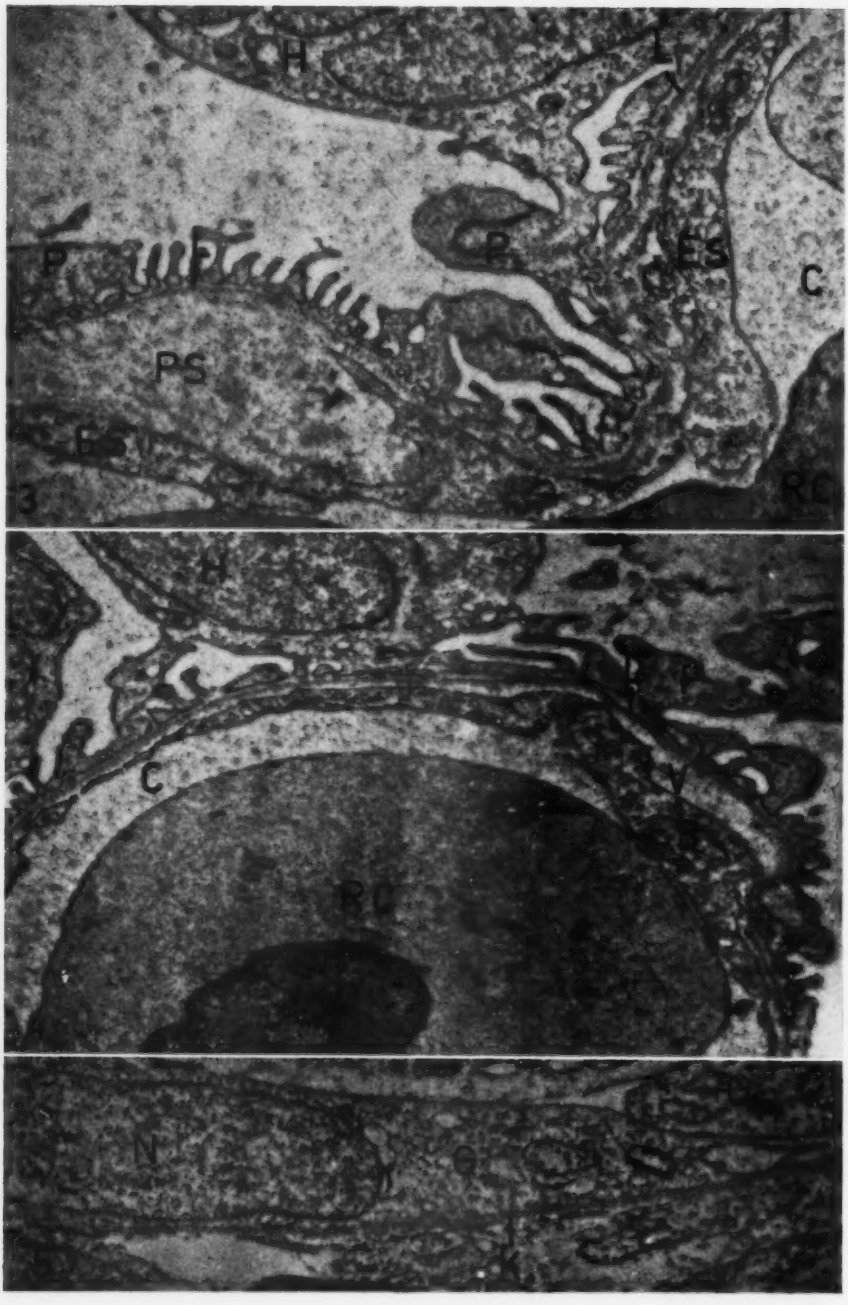
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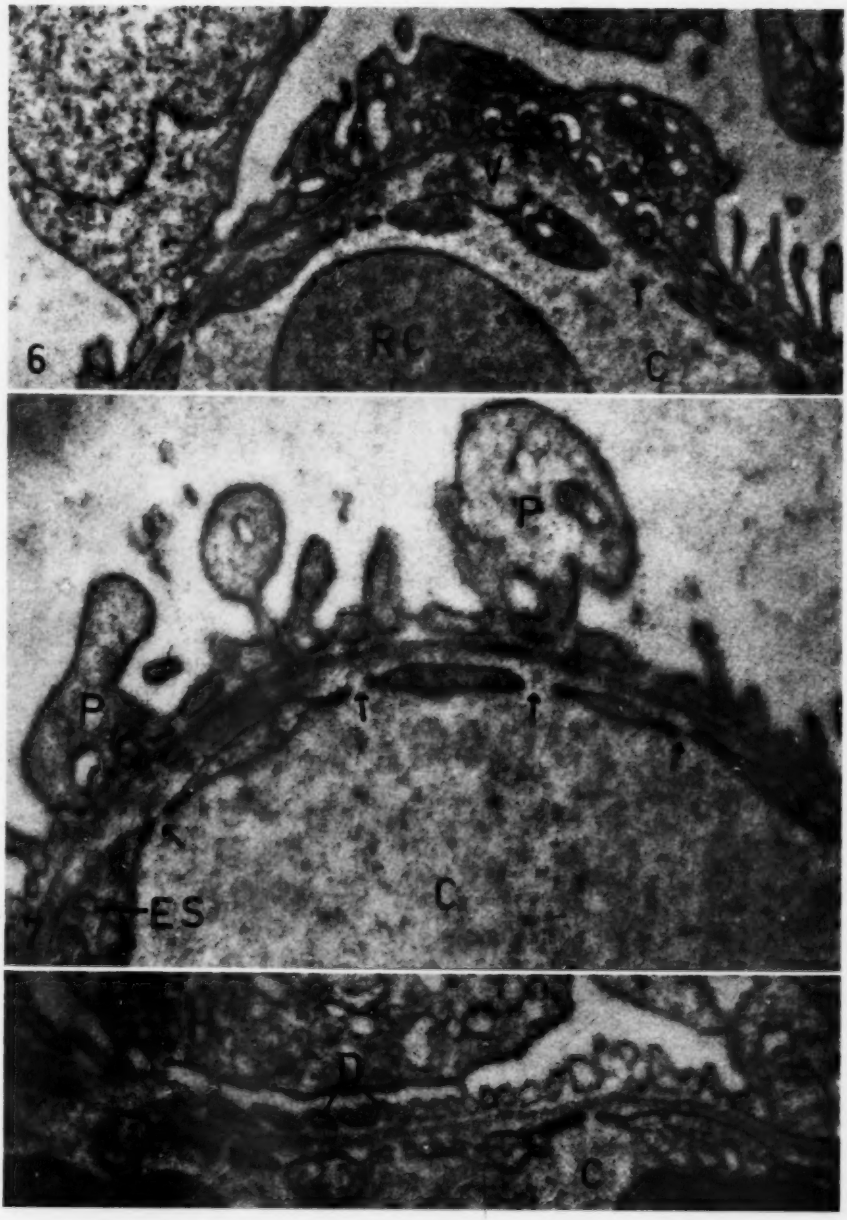
6
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Fig. 9. The electron micrograph shows an epithelial cell with its nucleus (Y) and its cytoplasm (P) lying for a large distance flat on the three-layered basement membrane (T). The capillary lumen (C) is lined by endothelial cytoplasm (ES). × 27,000.

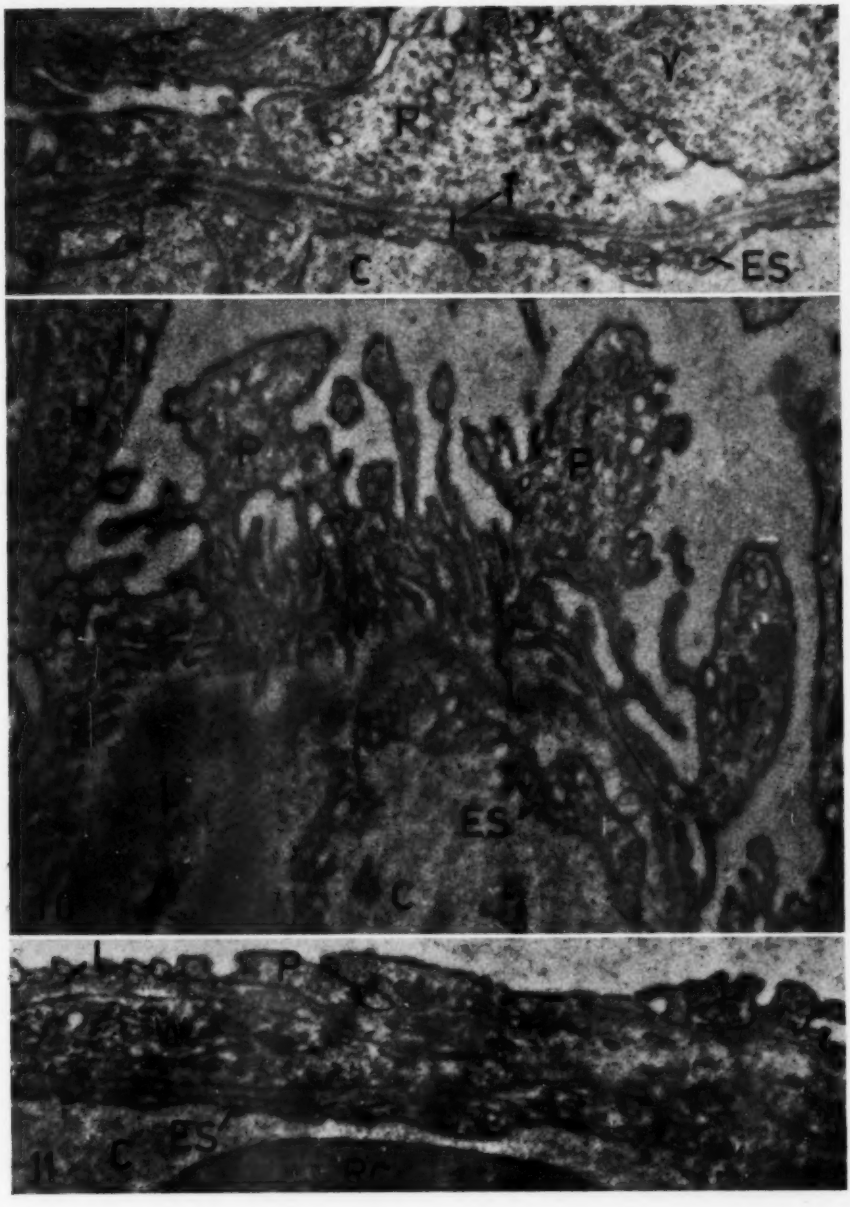
Fig. 10. The electron micrograph shows an epithelial cell (H) and pedicels (P). Tangential sectioning through the capillary wall shows an intricate pattern of pedicels at (J) and a wide lamina densa (L) of the basement membrane. The capillary lumen is at (C) and is lined by endothelium (ES). × 30,000.

Fig. 11. The electron micrograph shows the periphery of a capillary loop with a projection of cytoplasm (W) in the pericapillary space between the lamina densa (L) of the basement membrane and the attenuated endothelial sheet of cytoplasm (ES). Pedicels are present at (P) and a red cell (RC) is present in the capillary lumen (C). × 33,000.

Fig. 12. The electron micrograph shows the nuclei of three "intercapillary" cells (X) with their cytoplasmic body containing endoplasmic reticulum, dense granules and mitochondria and no distinguishing features except their position. Cytoplasmic projections without nuclei are present at (Z). Intercellular material (O) with small projections of cytoplasm fills the spaces between cell bodies. The major portion of the lamina densa (L) can be seen to run around the "intercapillary" tissue. An epithelial cell (H) projects onto the "intercapillary" tissue. Pedicels (P) can easily be recognised in Bowman's space. Capillary lumen (C) lined the endothelium (ES) and containing red cells (RC) are on the right side. "Intercapillary" tissue can be seen to spread into the peripheral wall of the capillary loop to form pericapillary tissue (W) in the pericapillary space. × 25,000.

Fig. 13. The electron micrograph shows the three layers (E), (L) and (U) which have been described as forming the basement membrane of the capillary loop. It is suggested that only the dense layer (L) should be regarded as the basement membrane. The external less dense layer is relatively constant in its relationship to the pedicels (P) and the dense layer (L). The internal less dense layer is extremely variable as shown in other electron micrographs. The capillary lumen (C) is lined by endothelium (ES) and contains a portion of a red cell (RC). × 32,000.

Fig. 14. The electron micrograph shows clearly the continuity of Bowman's capsular basement membrane (DL) with the dense layer (L) of the capillary basement membrane. In the region of the adventitial coat of the arteriole (M), Bowman's capsular basement membrane thickens a little as it joins up with the intercellular material of the adventitial coat and not necessarily if at all with the sub-endothelial basement membrane of the arteriole. The capillary lumen is at (C) while endothelial cytoplasm is designated (EC). A flattened layer of Bowman's capsular cell (MC) is in some areas separated from the basement membrane (DL) by a less dense layer. Epithelial pedicels are at (P) and the pale cytoplasm of the other type of cell which is present in Bowman's space and appears to rise from Bowman's capsular cells is at (F). × 30,000.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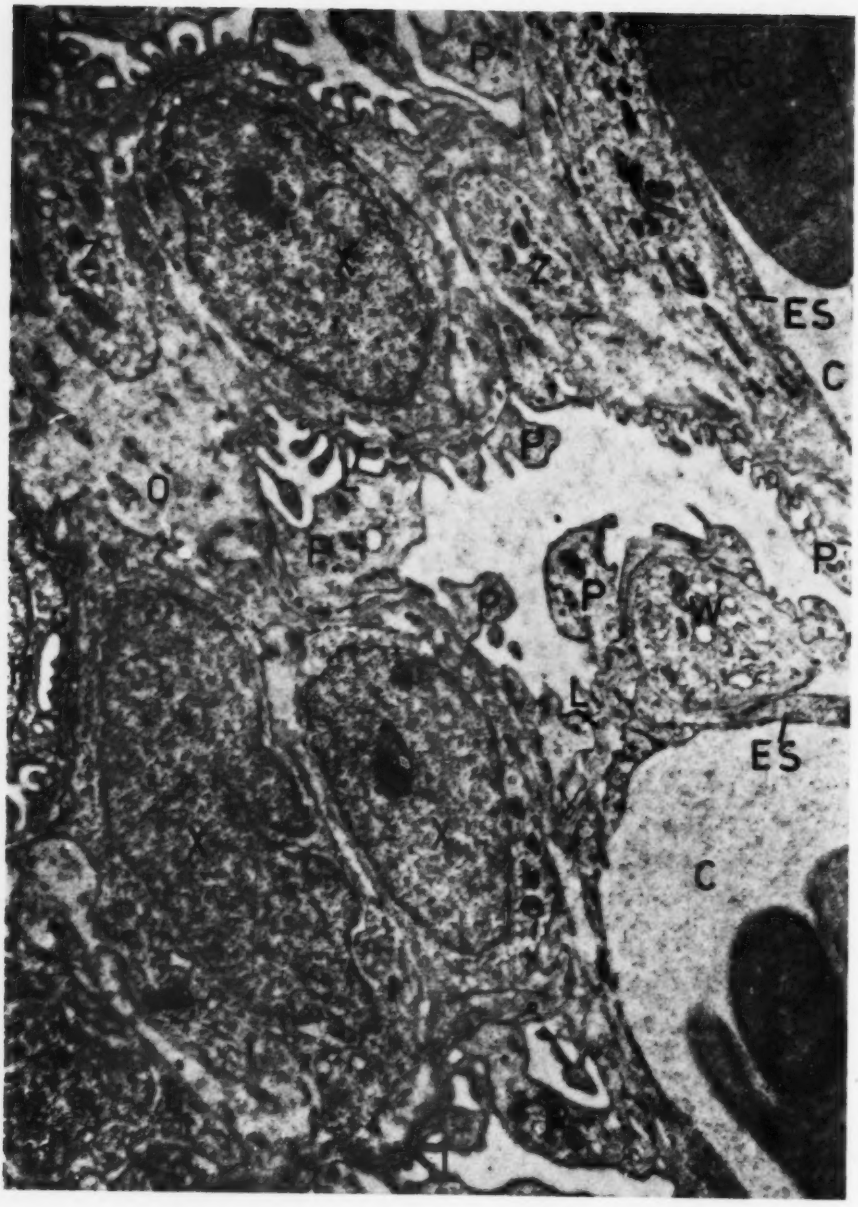
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Fig. 15. The electron micrograph shows an interesting variation (K) of the attenuated endothelial layer (ES). The material in the space so formed has the same electron density as the material (serum) in the capillary lumen (C). An epithelial cell (H) and pedicels (P) are in Bowman's space. × 32,000.

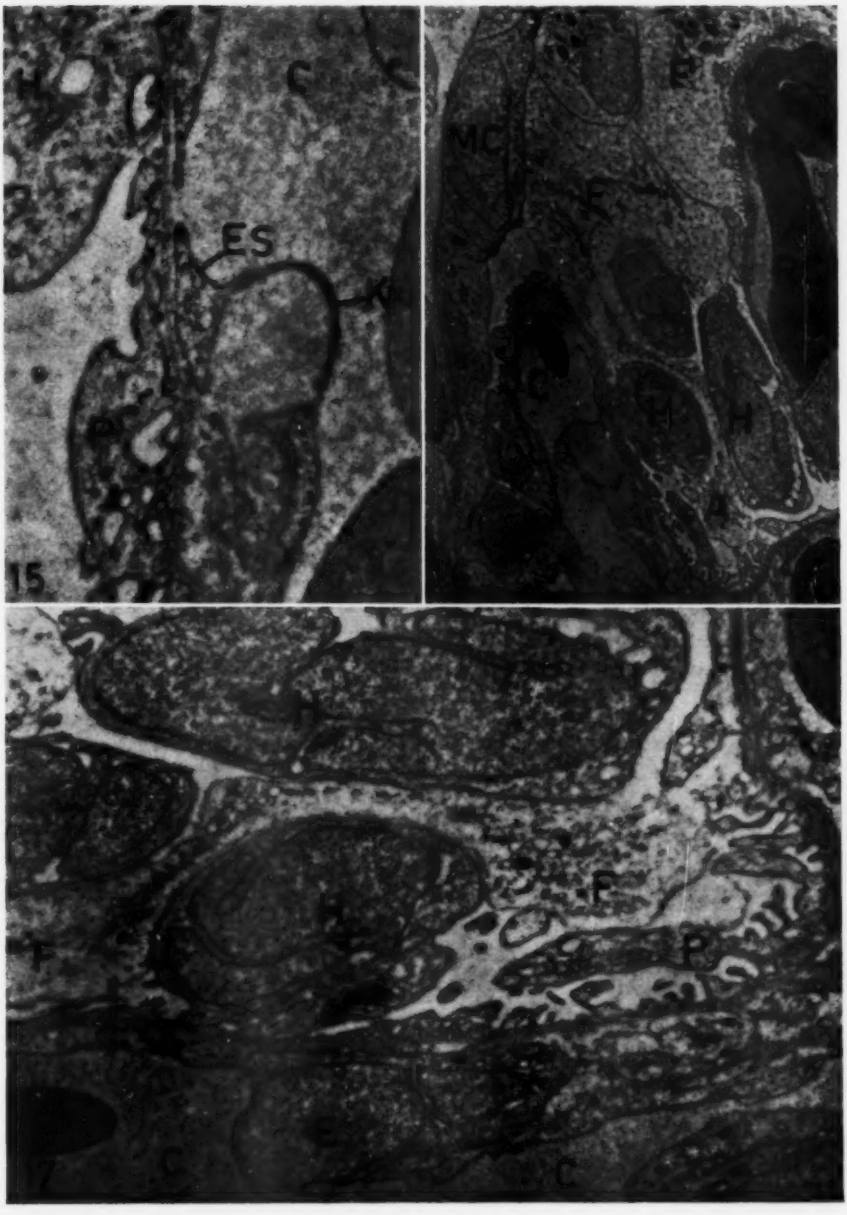
Fig. 16. This lower power electron micrograph shows the pale cell (F) which appears to rise from Bowman's capsular cells (MC), flowing between the epithelial cells (H). Capillary lumen are shown at (C) and red cells are designated (RC). In the region of (X) is the hilum of the glomerulus. The area designated (A) will be enlarged in Fig. 17. × 4,500.

Fig. 17. This electron micrograph is an enlargement of area (A) in Fig. 16. The pale cytoplasm can be seen flowing between epithelial cells (H) and pedicels (P), and on the right side the cytoplasmic membrane of the portion (F) is clearly visible in the lower edge, but in the upper area it fades into the material present in Bowman's space. Capillary lumen (C) is lined by endothelium (E). × 19,000.

Fig. 18. The electron micrograph shows the two types of cells lining Bowman's capsule. There is the large cell with pale cytoplasm (PC) and normal density nucleus (N) and there are cells with their cytoplasm (MC) having the same electron density as epithelial cells with nucleus (NM) having the same structural appearance as epithelial cell nucleus. Bowman's capsular basement membrane (D) separates the glomerulus from the peri-glomerular connective tissue (FB) which is probably cytoplasm of a fibroblast. Bowman's space is at (S). The morphological relationship between the two types of cells is clearly visible and their cytoplasmic membranes are separated by a very narrow space of intercellular material. × 22,000.

Fig. 19. The electron micrograph shows the nucleus of an endothelial cell (E) with its double nuclear membrane and the cytoplasm (ES) which lines the capillary lumen (C). A pericapillary space is at (V) and in the region of this letter the usual clear demarcation of the endothelial cytoplasm is lost. If the dense layer (L) is to be regarded as the true basement membrane corresponding to that of the light microscopist then it appears from this electron micrograph that it is more intimately related with the epithelial layer than the endothelial. Pedicels are at (P). × 23,000.

Fig. 20. The electron micrograph shows the neck of a glomerulus with the tubular lumen (TL) running in the direction of the arrow and is lined by tubular cells (TU). The two types of cells lining Bowman's capsule are at (F) and (MC). Direct continuity of Bowman's capsular basement membrane (D) with the tubular basement membrane is clear. Bowman's space is represented at (S) capillary lumen at (C) and an epithelial cell (H) projects onto the wall of a capillary at the opening of the neck. × 15,000.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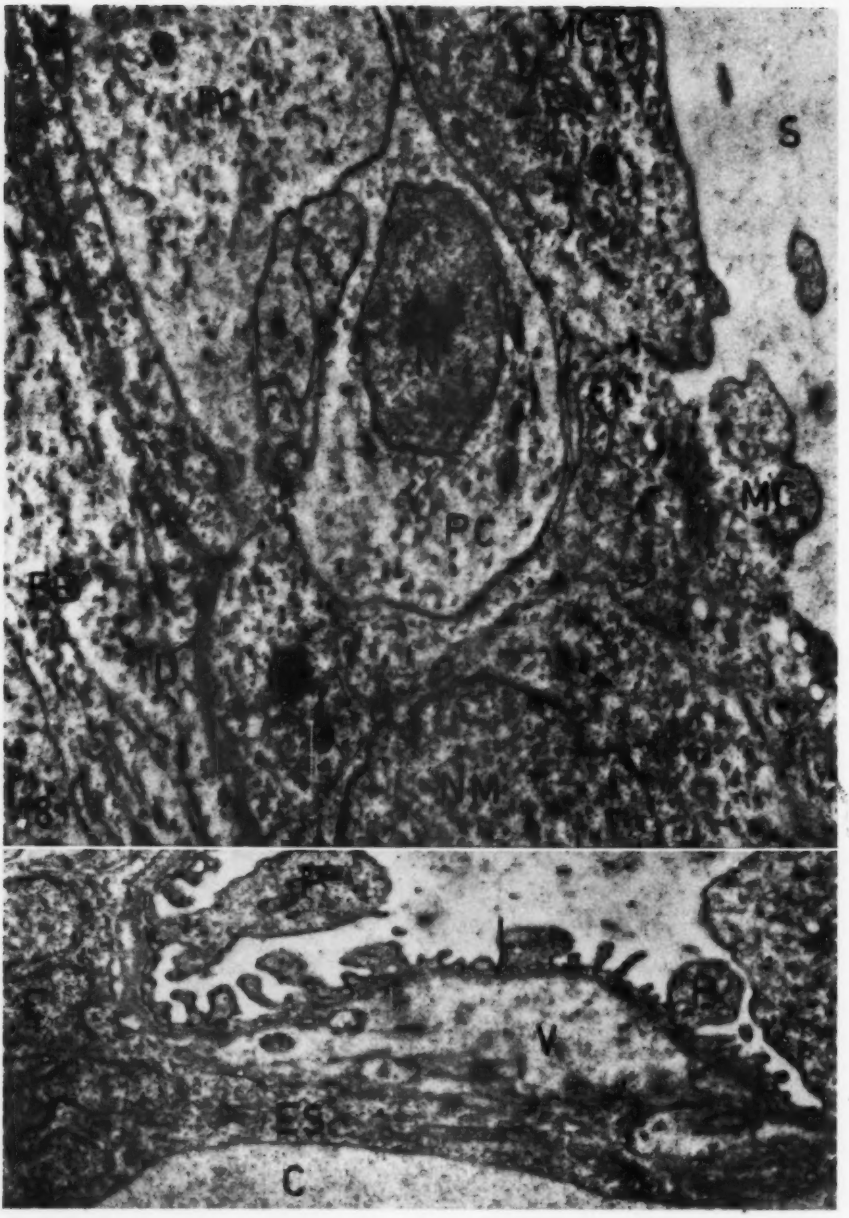
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The cytoplasmic membrane is relatively smooth and free of any invaginations such as are described in proximal convoluted tubules in mammals by Sjostrand and Rhodin (1953), and by Ruska *et al.* (1957). By contrast the second type of cell is very pale with remarkably poor electron density. The nucleus has the same characteristics as in the first type of cell, but the cytoplasm contains only a small quantity of endoplasmic reticulum, dense granules and mitochondria or none at all (Fig. 18). The cytoplasmic membrane is well defined. These two types of cells line Bowman's capsule either as a single thin flattened layer of cytoplasm or as a double layer of cytoplasm in which tall cuboidal cells are present (Fig. 18). There is no particular distribution or arrangement, but the cell surfaces often invaginate each other (Fig. 18). Adjacent cytoplasmic membranes are close together with very little intercellular space between them. Bowman's capsular cells lie on a thin dense single layer of intercellular material (basement membrane) from which it may be separated in some areas by a less dense layer.

Hilum. At the hilum there is direct continuity between Bowman's capsular basement membrane with its less dense layer, and the lamina densa and lamina rara externa respectively (Fig. 14). It is apparent that the major portion of the capillary loop basement membrane is continuous with Bowman's capsular basement membrane.

Neck. In this region there is direct continuity between Bowman's capsular cells and the tubular cells and also the basement membrane is continuous between Bowman's capsule and the tubule (Fig. 20).

DISCUSSION.

The mesonephric glomerulus of the common goldfish has features very similar to those found in the amphibian mesonephric glomerulus, viz., sub-epithelial capillary loop basement membrane in some areas, haphazard distribution of endothelial cells, pericapillary tissue, drop-like terminal foot processes, and large flattened terminal foot processes, but it also possesses the three layered capillary loop basement membrane described in higher vertebrates and a more complex epithelial pedicellar arrangement. It is possible that the piscine mesonephric glomerulus may represent an intermediate stage in the degree of glomerular specialisation.

From a comparison of light and electron microscopic studies it now seems justifiable to conclude that the basement membrane of the light microscopist, is represented by an electron-dense layer in electron microscopical studies such as those on the choroid plexus (Millen and Rogers, 1956; Maxwell and Pease, 1956), the placenta (Dempsey and Wislocki, 1956), the thyroid gland (Ekholm and Sjostrand, 1957) and the renal tubule (Sjostrand and Rhodin, 1953). In adopting this view, the lamina densa of the previously described three-layered glomerular capillary loop basement membrane becomes the "true" basement

membrane in electron microscopical studies and the two less dense layers, lamina rara externa and interna (Yamada, 1955), become layers of intercellular material which may have a cementing characteristic as suggested by Pease (1955).

The lamina rara externa is a reasonably constant structure, although now and then it is obliterated when epithelial pedicels are in contact with the lamina densa. The lamina rara interna is a more variable structure which widens considerably in the piscine and amphibian mesonephric glomeruli to form the pericapillary space which may contain pericapillary tissue. However, in the bird (Pak Poy and Robertson, 1957) and in the rat (unpublished data) it may become obliterated by the attachment of the endothelial layer to the lamina densa. Because of the extreme variability it is unlikely that its function is purely cementing in nature, but more possibly it is a labile layer of intercellular material, which may play an important part in pathological lesions of the glomerulus. Reid (1956) considered that in advanced lesions of nephrotoxic serum nephritis, the focal osmic-stained deposit was situated in this space.

There is little doubt that in the amphibian and piscine mesonephric glomeruli the major portion of the lamina densa ("true" basement membrane) of the capillary loop basement membrane is continuous with Bowman's capsular basement membrane. The lamina rara externa is continuous with the less dense layer (whenever this layer is present), which separates Bowman's capsular cells from the dense basement membrane layer. Frequently this less dense layer, unlike that of the capillary loop, is absent. When the connective tissue surrounding a glomerulus is torn away artefactually the dense basement membrane remains attached to the Bowman's capsular cells and frequently when pericapillary tissue is present the lamina densa of the capillary loop basement membrane remains attached to the epithelial pedicels. This firm attachment of the basement membrane to the epithelial and Bowman's capsular cells suggests that the basement membrane is more closely related to the epithelial cell than to the endothelial cell.

Tall cuboidal cells have been described as lining Bowman's capsule in the kidney of the mouse, dog, mink and the woodchuck by Hemholz (1935) and in the American opossum by McNider (1927), and Crabtree (1941) has suggested that in mice there is a numerical relationship between their presence and the age and sex of the animal. These cells have been described as spreading from the neck of the glomerulus around to the equator and even to the hilum of the glomerulus. In the goldfish's glomerulus these cuboidal cells appear to be scattered haphazardly. Edwards and Schnitter (1933), although they do not describe cuboidal cells, published a photomicrograph of a renal glomerulus of a goldfish in which cuboidal cells are present in Bowman's capsule. I have not been able to find any references in the literature to a double layer of cytoplasm lining Bowman's capsule in any animal. Edwards and Schnitter (1933)

described the presence of dark homogenous bodies throughout the tubule in both marine and fresh water teleosts. No attempt has been made to correlate these two factors as yet. The finding of large masses of pale cytoplasm between epithelial cells is inexplicable in this study.

In conclusion, therefore, mesonephric glomeruli exhibit certain characteristic features: (1) The presence of pericapillary tissue; (2) The close relationship of the dense middle layer of the capillary loop basement membrane (lamina densa) with the epithelial cell; (3) The major portion of the capillary loop basement membrane is continuous with Bowman's capsular basement membrane; (4) The presence of drop-like and large flattened foot processes rather similar to those described in lipoid nephrosis in man (Farquhar, 1957); and (5) Haphazard distribution of endothelial cells.

SUMMARY.

Electron microscopical study of the mesonephric glomerulus of the common fresh water goldfish shows features similar to those described in the mesonephric glomerulus of the frog. These features are as follows: (1) The presence of pericapillary tissue; (2) The close relationship of the lamina densa with the epithelial cell; (3) The major portion of the capillary loop basement membrane is continuous with Bowman's capsular basement membrane; (4) The presence of drop-like and flattened epithelial foot processes; and (5) Haphazard distribution of endothelial cells. Two types of Bowman's capsular cells are present. It is suggested that the lamina densa of the three-layered capillary loop basement membrane be regarded as the true basement membrane.

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OBSERVATIONS ON DRYING BACTERIA FROM THE FROZEN AND FROM THE LIQUID STATE[1]

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The two well-established methods of drying microorganisms for purposes of preservation are (1) drying at temperatures below 0° C. from prefrozen material (F drying) and (2) centrifugal (evaporative) freeze drying (C drying). In a previous paper (Annear, 1956) it was suggested that more attention might be paid to the investigation of drying suspensions from the liquid state (L drying).

Stamp (1947) has shown that reasonable recoveries of some bacteria may be obtained by *slow* drying from the liquid state. The method was, however, unsatisfactory for 'sensitive' organisms such as *Vibrio cholerae*.

In the present study, organisms have been dried *rapidly* from the liquid state and direct comparisons have been made with C and F methods of drying. Drying from the liquid state has been assured by supplying sufficient heat to the suspensions to prevent their freezing during evaporation *in vacuo*.

The test organism for most of the experiments was a salmonella, but two small L drying experiments were also done with a vibrio.

The organisms have been dried in mixtures of peptone and glucose and the effect of glucose concentration has been studied in all three drying methods.

With both L and F methods, the organisms were dried in small volume aliquots (0·04 ml. or less) while with the C method, the volumes dried were 0·2 ml.

The experimental conditions in many of the F dryings were such that desiccation did not in fact take place continuously from the frozen state and this consideration has been important in assessing the results.

The investigations have been concerned for the most part with recoveries during or immediately after drying, but several brief term storage results are also reported.

Methods.

Organisms.

The two organisms were *Salmonella ndolo* (N.C.T.C. 8700) and *Vibrio cholerae* (624). The latter organism was used extensively in a previous study (Annear, 1956).

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[1] This work was carried out during the tenure of a John Lucas Walker studentship.

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Preparation and dispensing of suspensions for drying.

The organisms were dried in either (A) a mixture of Evans peptone and glucose (in equal concentration) or (B) mixtures of peptone (4 p.c.) with varying concentrations of glucose (0-24 p.c.).

Light suspensions of the organisms were first made in nutrient broth from a nutrient agar plate culture which had been incubated at 30° C. for 20 hours, then a 1/250 dilution of this suspension was made in the test suspending fluid.

Ampoules of 8 ml. capacity and of the type shown in Fig. 1 were used for F and L drying while tubes (4 in. × 5/16 in.) were used for C drying. A 'syringe-pipette' (Annear, 1956) which dispensed drops of constant volume was used for charging the ampoules. The number of organisms per ampoule or tube dried was of the order of 10^4 to 10^5.

"A" mixture. For the L and F methods of drying, the ampoules were each charged with one drop of the bacterial suspension in the "A" mixture. For the C method, the tubes were first charged with ten drops of sterile "A" mixture and then with single drops of the bacterial suspension in the same mixture. Thus equal numbers of organisms could be dried in parallel by all three methods.

"B" mixture. For L and F drying, the ampoules were first charged with single drops of sterile glucose solutions (double the concentration of that finally required) then inoculated with single drops of the organism suspended in 8 p.c. peptone. For the C method, the tubes were charged with ten drops of the sterile glucose-peptone solutions then inoculated with single drops of the organism suspended in 4 p.c. peptone. Variations in the 'drop volumes' of the various suspending fluids were allowed for in calculating the final concentrations of the solutes in the mixtures.

Drying methods.

For L and F drying, flask manifolds (Fig. 1) which could accommodate as many as 12 ampoules were used. These manifolds were connected to a central manifold which was mounted over a P_2O_5 trap and connected to a high vacuum pump (Annear, 1956; Annear, 1958). The path from the evaporating surface to the P_2O_5 in the flasks was reasonably short and direct, the ampoule itself being a limiting factor in the assembly.

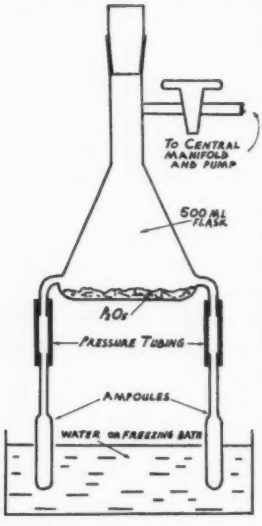

Fig. 1. Diagram of assembly used for F and L drying.

With respect to L drying, whether with the vacuum assembly used here, small volumes of water would freeze or not during evaporation was shown to depend upon the heat supplied. If, for example, the ampoules were surrounded by ordinary room air (at, say, 20° C.) the water froze and continued to evaporate from the frozen state. If, however, the ampoules were held in a water bath at the same temperature or higher, freezing was prevented by the ready transfer of heat from the water bath to the water in the ampoule, which evaporated very rapidly. The presence of soluble material in the evaporating liquid further inhibits freezing. For the experiments with *S. ndolo*, the treatment given was immersion in a water bath at 20° C. for 10 minutes; for the two experiments with *V. cholerae*, 30° C. for three minutes. The tubes were not exposed to high vacuum until they were immersed in the water baths. As far as could be judged by eye, the suspensions were dried within two minutes by the first treatment and within one minute by the second. The baths were then removed and drying was continued for periods stated in the experiment data (at least 24 hours).

For F drying, the ampoules, after being charged with suspension, were immersed in a bath at −75° C. for one minute then transferred to the bath of the temperature at which drying was to be carried out. These temperatures (−5, −8, −12, −23 and −40° C.) were obtained with solid CO_2 and various organic solvents. Drying was carried out for periods stated in the experimental data. In some experiments, the ampoules were then brought to room temperature and drying was continued for a further period. Counts were made either after the low temperature drying or after the total drying treatment or in some experiments after each treatment.

C drying was done in an Edwards L5 unit. The tubes were held in the primary chamber for six hours then transferred to the central manifold where drying was continued for periods stated in the experimental data.

Storage of desiccates.

Ampoules or tubes which were to be stored were constricted (as a prerequisite to sealing off) during the last few hours of their period on the manifold. The desiccates were stored in darkness at room temperature.

Viable counts.

The general procedures for making counts have been described (Annear, 1956). All counts were made on blood agar and the diluent was nutrient broth.

Periodic counts were made in each experiment in order to check the stability of the suspensions for the period during which the experiment was put up. In those experiments where the effect of glucose concentration was studied, the organisms were exposed to as many as eight different media. The peptone suspension of the organisms was counted at the beginning of the experiment and again immediately after the manipulations with the manifolds were finished, i.e., when the suspensions were undergoing drying. Also at this time one ampoule of each glucose-peptone suspension (retained for the purpose) was counted. There were in some experiments therefore as many as ten suspensions to provide a mean for the initial number of viable organisms per ampoule. In none of the experiments described here were there significant differences between the 'zero' counts. The longest period taken to put up an experiment including the counts was about 30 minutes.

For almost all treatments, the ampoules were examined in batches of three.

Appearance of the Desiccates.

For purposes of discussion some terms for describing the appearance of the desiccates are defined below.

Desiccates of the F method.

General considerations. The period required for dehydrating material from the frozen state depends upon the efficiency of the vacuum assembly, the temperature of the frozen material and the nature and concentration of the solutes in it. Information on this question was obtained only by trial and error and by visual inspection of the dried material. In the majority of experiments where F drying was done, the suspending medium was a mixture of 7 p.c. peptone and 7 p.c. glucose. In preliminary observations a 2-drop volume of this mixture was frozen at −75° C. then dried at −40° C. for 24 hours. The ampoule was then clipped off and stoppered while it was still immersed at −40° C. Examination of this material at room temperature showed it to powder readily when the ampoule was jarred, and to show no sign of melting when the ampoule was warmed to 55° C. In view of the extremely deliquescent nature of these glucose-peptone mixtures the above observations were taken to indicate a high degree of dehydration of the freeze dried material. In the experiments described in this paper (with 7 p.c. glucose and 7 p.c. peptone), −40° C. was the lowest temperature at which material was dried, 24 hours was the shortest drying period

and the largest quantities dried were one-drop volumes, thus ensuring a considerable margin for dehydration. It is emphasised that these one-drop volumes occupied a very shallow layer over the bottoms of the ampoules thus exposing large surfaces for drying.

The foregoing considerations are pertinent in the descriptions of the F desiccates.

'Powdery'. Suspensions which were dried at a sufficiently low temperature and for a sufficiently long period yielded a whitish homogeneous shallow saucer of material. No change in appearance occurred when such a desiccate, still under vacuum, was raised from the freezing bath to room temperature.

'Bubbly'. Where the temperature of the bath was too high to keep the suspension frozen during drying, the product was pitted and 'bubbly' in texture.

'Puffy'. With high concentrations of glucose and a sufficiently low drying temperature, the material remained frozen during drying; but if such material, still under vacuum, was raised to room temperature before sufficient water was removed, the partially dried 'saucer' thawed and puffed into a compact dome-shaped structure in which form it set and dried rapidly. The thawing was extremely transient and the material passed so rapidly from the frozen state to the solid dry state that it remained opaque during the process. The material was thus finally dried from a viscous state. Such desiccates occurred in only one experiment (last three points in the F curve of Fig. 2).

Desiccates of the C method.

'Smooth'. Where the glucose content of the suspensions was nil or sufficiently low, the material dried as a smooth wedge in the tube. All such desiccates had, however, a surface skin and were finely vacuolated; they were never as homogeneous as the 'powdery' desiccates of the F method.

'Foamy'. With higher glucose concentrations the material although freezing initially thawed partially during drying and finished up as a 'foamy' looking product. Such desiccates occurred in only one experiment (last two points in the C curve of Fig. 2).

Desiccates of the L method.

'Scaly'. Almost all of the suspensions dried from the liquid state, dried as scales strongly adherent to and protruding from the rounded bottom of the ampoule.

'Glassy'. Occasionally, and for as yet no known reason, the suspension dried as a glassy almost invisible film on the rounded bottom of the ampoule. The concentration of glucose in the suspensions did not appear to affect the structure of the material dried by the L method as it did with the others.

EXPERIMENTAL.

The effect of glucose in the suspending medium.

With the C and L drying methods (Fig. 2) there was a direct relationship between glucose concentration and recovery after drying. With the C method, the desiccates containing the two highest glucose contents were 'foamy' while those with the lower glucose concentrations were 'smooth'. In peptone alone, the recoveries with the L drying were about twice those of the C method.

The relationship between glucose concentration and recovery with the F method (Fig. 2) was a more complex one with a curious sharp peak at about 2 p.c. glucose and a plateau of high recoveries above 14 p.c. glucose. A point of considerable interest here was that the desiccates associated with this plateau were all 'puffy', whereas those of the lower concentrations were 'powdery'.

Each of the three experiments shown in Fig. 2 was done on a different suspension of organisms so the results are not strictly comparable. It is apparent, however, that all three methods yield high recoveries of the organism between some limits of glucose concentration and that these limits vary with the method.

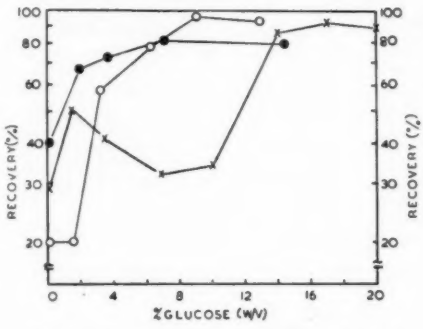

Fig. 2. Recoveries of *Salmonella ndolo* after drying in 4 p.c. peptone plus various concentrations of glucose. Each point is the mean of three observations.
●—● L drying: + 20° C. bath for 10 min., then room atmosphere for 48 hours.
×—× F drying: — 23° C. for 72 hours, then room atmosphere for 24 hours.
○—○ C drying: Primary chamber for 6 hours, then room atmosphere for 42 hours.

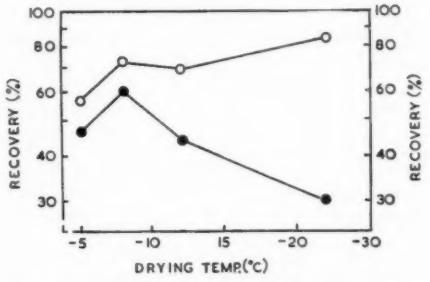

Fig. 3. Recoveries of *Salmonella ndolo* after drying at various temperatures (F method) in 7 p.c. peptone + 7 p.c. glucose. Each point is the mean of three observations.
○—○ Recoveries after drying for 48 hours at temperature shown.
●—● Recoveries after drying for 48 hours at temperature shown followed by drying for 24 hours in room atmosphere.

Effect of drying temperature in the F method.

Fig. 3 shows results from an experiment in which the organisms were dried in a medium of constant composition and at four different temperatures for 24 hours followed by drying at room temperature for 24 hours. Counts made at the end of the first part of the drying revealed highest recovery from the tubes dried at the lowest temperature (— 23° C.). After further drying at room temperature, however, a large fraction of the remaining organisms was lost and at this stage of the experiment, the highest recoveries were obtained in those desiccates which had been dried initially at — 8° C. The desiccates dried at — 23° C. were 'powdery', whereas those dried at the higher temperatures were progressively more 'bubbly'. No change in appearance took place with any of the desiccates after they were raised to room temperature.

Similar results may be seen in Table 1 (Exp. 733, drying treatments 3, 4 and 5). Also in this experiment, recoveries after a short storage revealed stable preservation of those desiccates dried initially at — 8° C. and substantial further losses in those dried at the two lower temperatures.

TABLE 1.

Recoveries of Salmonella ndolo *after drying and storage.*

Suspending medium: 7 p.c. glucose +7 p.c. peptone.

Exp.	Drying treatment	Recovery p.c.	
		At end of drying treatment	After storage for 5 months
731	1	85, 76, 83 (81)	66, 72, 75 (71)
	3	49, 43, 50 (47)	18, 19, 20 (19)
732	1	74, 75, 79 (76)	67, 79, 88 (78)
	3	53, 49, 48 (50)	19, 17, 17 (17)
733	1	84, 86, 79 (83)	82, 62, 75 (73)
	3	56, 56, 51 (55)	25, 24, 29 (26)
	4	51, 54, 48 (51)	13, 8, 8 (13)
	5	81, 83, 83 (83)	97, 95, 93 (92)
	6	101, 104, 100 (102)	101, 99, 92 (97)

Drying treatments.

1. L drying: 10 min. in +20° C. bath then room atmosphere for 48 hr.
3. F drying: −23° C. for 24 hr., then room atmosphere for 24 hr.
4. F drying: −40° C. for 24 hr., then room atmosphere for 24 hr.
5. F drying: −8° C. for 24 hr., then room atmosphere for 24 hr.
6. C drying: Primary chamber for 6 hr., then room atmosphere for 42 hr.

Comparison of drying methods.

In an experiment (Table 2) where one suspension was dried by the three methods in parallel, there was little difference between the recovery for the L and C methods and the recoveries were stable during five days of drying. With

TABLE 2.

Recoveries of Salmonella ndolo *after various drying treatments in a single experiment.*

Suspending medium: 7 p.c. peptone +7 p.c. glucose.

Drying treatment	Recovery p.c. after drying for		
	24 hr.	48 hr.	120 hr.
(1) L drying: +20° C. bath for 10 min., then room atmosphere for periods stated	76, 90, 67 (78)	89, 73, 78 (80)	70, 72, 81 (74)
(2) C drying: Primary chamber for 6 hr. then room atmosphere for period stated (less 6 hr.)	—	83, 85, 82 (83)	100, 85, 85 (90)
(3) F drying: −23° C. for periods stated	93, 86, 86 (88)	80, 83 (81)	73, 84 (78)
(4) F drying: −23° C. for 72 hr. then room atmosphere for 48 hr., (i.e. 120 hr. in all)	—	—	56, 62 (59)

the F method, the usual loss occurred when the ampoules were taken from —23° C. to room temperature. Prolonged drying at — 23° C. for five days, however, resulted in little if any loss after the initial small fall.

Table 1 also shows results from several direct comparisons of the drying methods. No losses were detected for the C method in Exp. 733 (treatment 6). In all three experiments, fairly high recoveries (about 75 p.c.) were obtained in the L drying method. Also in all three methods the desiccates dried by treatment 3 (drying at — 23° C. for 24 hours followed by drying at room temperature for 24 hours) showed an approximate 50 p.c. loss with further heavy losses after storage. Exp. 733 is of interest because it shows high recoveries by all three methods of drying (treatments 1, 5 and 6).

Irregularities among desiccates dried by the L method.

As stated earlier in the text, two distinct types of desiccates resulted from L drying; the more usual was 'flaky' or 'scaly' in appearance and the less usual, a transparent 'glassy' film on the wall of the ampoule. It was also observed that occasionally a divergent count or counts occurred among replicate ampoules and the suspicion arose that there might be some relationship between the recoveries and the physical nature of the L desiccates. Note was then taken of the appearance of each desiccate before the count was made. Also one experiment was put up specifically to test this point. Three of the twelve resultant desiccates were 'glassy'. Of the remaining nine 'scaly' desiccates, three were picked at random and counts were made and compared with those from the three 'glassy' desiccates. The results from this experiment and from the other observations are shown in Table 3. Although there were not a large number of comparisons available, the results were most consistent in showing that the lower recoveries were associated with the 'glassy' desiccates and the higher recoveries with the 'scaly' desiccates.

TABLE 3.

Recoveries of Salmonella ndolo *from 'scaly' and from 'glassy' desiccates dried by L method.*
Suspending medium: 7 p.c. peptone +7 p.c. glucose.
Drying: +20° C. bath for 10 min., then room atmosphere for 24 hr.

Nature of Desiccate	Recovery p.c.					
	Exp. 1	2	3	4	5	6
'Scaly'	81, 94, 95	90	89, 78	81	75	74
'Glassy'	77, 74, 71	76, 67	73	70, 72	56, 62	53, 54

Recoveries of Vibrio cholerae *dried by the L method.*

The results of two experiments in which *V. cholerae* was dried by the L method are shown in Table 4. Apart from one divergent count in Exp. 700, the

results are consistent and show an immediate recovery of about 30 p.c. and a further fall to approximately half that value after storage for six months.

TABLE 4.

Recoveries of Vibrio cholerae *after L drying and storage.*

Suspending medium: 5 p.c. peptone +5 p.c. glucose.

Drying: +30° C. bath for 3 min., then room atmosphere for 24 hr.

Exp.	Recovery p.c.	
	At end of drying period	After storage for 6 months
700	37, 17, 41 (32)	19, 17, 17 (18)
701	31, 36, 28 (32)	19, 22, 23 (21)

DISCUSSION.

F method of drying.

The results obtained with this method of drying have depended upon a number of factors. Where drying was completed at room temperature, high recoveries were obtained only in material which was not properly frozen during drying, i.e., in material which either thawed during the low temperature dehydration (Fig. 3, —8° C. and Table 1, treatment 5) or which thawed, when raised still under vacuum, to room temperature (last three points on the F curve of Fig. 1). There may be some conditions under which high survival is associated with a 'properly' freeze-dried mixture of glucose and peptone, but as yet they have not been discovered.

Greaves (1956) obtained an 80 p.c. recovery of a paracolon bacillus which was dried by an F method in peptone and glucose, and it can be inferred from his remarks that the mixture did not dry as a homogeneous desiccate in his machine. The inclusion of dextran in the mixture, however, resulted in a "beautifully" dried product, and also a high recovery (93 p.c.) of the organism. High recoveries with F methods of drying and other suspending media have also been obtained by Naylor and Smith (1946) and by Wolff (1952), but these authors do not mention the appearance of their desiccates.

The fall in recoveries which occurs when suspensions dried at low temperature are raised, still under vacuum, to room temperature (Table 2, treatments 3 and 4 and Fig. 3, —23° C. point) poses an interesting problem. It is perhaps possible that the finely divided homogeneous powder which results from drying at low temperatures loses residual water readily when raised to a higher temperature and that 'overdrying' (Fry and Greaves, 1951) is responsible for the fall. If this is so, the same result might be obtained by prolonged dehydration at the lower temperatures. The results shown in Table 2 (treat-

ment 3) are inconclusive in this respect. Further work with more efficient means of removing water vapour at low temperatures might shed some light on this problem. By contrast, the higher recoveries which result from material not 'properly' freeze-dried may be due to the retention of water by these coarser structures.

The lower curve in Fig. 3 shows that for high recoveries with F drying there is an optimum temperature for the first stage of dehydration, when this stage is followed by drying at room temperature. It seems most likely that this optimum will vary with the concentration of the solutes in the suspending medium and will be such that some degree of thawing occurs during drying.

The effects of the sudden cooling of the suspensions to —75° C. and the 'warming' of them to the drying temperature (—40° to —5° C.) have not been studied *per se*, but it is axiomatic that the losses due to these treatments were no greater than the total losses after drying and these in many cases were negligible.

No explanation can yet be given for the rather intriguing peak near the 2 p.c. glucose level which occurred with F drying (Fig. 2). The experiment was repeated several times, always with similar results.

C method of drying.

Fry and Greaves (1951) showed that with C method of drying, glucose improved the recoveries of organisms dried in serum. Similar results have been shown here with peptone and there is enough evidence now to suggest this substance as a substitute for serum in the routine preservation of cultures by centrifugal freeze-drying.

A point of interest uncovered here was that for immediate survival at least, partial thawing of the initially frozen wedge during drying did not result in any measurable loss in recovery (last two points in C curve, Fig. 2). Such material has not yet been tested after storage. What storage results are to hand indicate that effective preservation is obtained after drying in a mixture of 7 p.c. peptone and 7 p.c. glucose, a medium which dried as a 'smooth' wedge, but not as a homogeneous powder. It seems a strong possibility therefore that with peptone and glucose at least, the high recoveries obtained with centrifugal freeze-drying are attributable to the relatively high temperature (—7° C., according to Greaves, 1956) at which the drying takes place.

L method of drying.

The results for this method show that it is possible to obtain high recoveries (sometimes more than 90 p.c.) of organisms from suspensions which are dried rapidly from the liquid state. The recoveries of *Vibrio cholerae* are particularly significant in this respect. It is most likely that the velocity of drying is an important factor in this technique and that slower drying (promoting longer exposure of the organisms to concentrating solutes) from the liquid state would result in a higher mortality.

Higher recoveries were obtained with glucose and peptone as a suspending medium than with peptone alone but the differences were not so great as they were with the other two methods.

It is apparent, that as with freeze-dried material, the viability of the organisms in desiccates dried by the L method varies with the physical form which the material assumes on drying. Whether survival is influenced by the structure of the desiccates *per se* or by associated factors, is not yet known. The rapid drying makes it very difficult to examine the problem from a dynamic point of view.

The explanation for the heterogeneity among the recoveries, whatever it is, might well be applicable to that which was associated with suspensions dried in ampoules externally exposed to room air (Annear, 1958).

General.

The experiments in this paper and in previous work (Annear, 1956; Annear, 1958) have shown that *Salmonella ndolo* and other organisms may be dried in peptone and glucose with little or no loss by treatments which vary widely with respect to the temperature of drying and the rate at which water vapour is removed.

A great deal of emphasis has been placed in the past upon the frozen state as an *essential* factor for the effective drying of organisms. The results obtained here in showing that high recoveries may be obtained from drying non-frozen material contradict this view.

Many of the desiccates in which high recoveries have been obtained could not be considered pharmaceutically elegant in appearance, a feature which is of some importance in projects such as B.C.G. immunization (Ungar, Farmer and Muggleton, 1956); they were, however, all freely soluble and quite suitable for purposes of maintaining stock cultures.

Of the three methods of drying described here, the L method is undoubtedly the simplest and cheapest and its effectiveness as far as has been tested is of the same order as the other two. It does, however, give more variable results and at present it must be considered more of academic interest than of practical value. For some bacteria at least, drying suspensions on cellulose tufts (Annear, 1958) would seem to be as simple but a more reliable method of preserving stock cultures.

SUMMARY.

Suspensions of *Salmonella ndolo* were made in mixtures of peptone and glucose and dried (a) from the liquid state, (b) by centrifugal freeze drying, and (c) at temperatures below 0° C. from prefrozen material. Relationships were shown to exist between the physical structure of the desiccates and the recoveries obtained from them. In particular, with the third of the above

methods, high recoveries were obtained only from material which thawed partially during drying. Glucose was shown to have a marked preservative effect with all three methods of drying.

The results showed generally that high recoveries of *Salmonella ndolo* could be obtained with a wide variety of drying conditions and recoveries of as high as 90 p.c. were obtained from desiccates dried from the liquid state.

Recoveries of about 30 p.c. were obtained after drying suspensions of *Vibrio cholerae* from the liquid state.

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THE UPTAKE OF ISONIAZID BY WASHED CELL SUSPENSIONS OF *MYCOBACTERIA* AND OTHER ORGANISMS

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The literature on the mode of action of isoniazid contains accounts of the uptake of radioactive isoniazid by cultures of *Mycobacteria* (Barclay, Ebert and Koch-Weser, 1953a, 1953b; Barclay, Koch-Weser and Ebert, 1954; Koch-Weser, Tricou, Barclay and Ebert, 1955) and of the inhibition by isoniazid of various biochemical systems, but no data are available on the uptake of isoniazid by washed cell suspensions which are often used in biochemical tests.

The following experiments show the effects of experimental conditions on the uptake of isoniazid by washed cell suspensions. Although the information obtained does not yet permit the formation of a theory on the mode of action of isoniazid, it is presented now for the bearing it has on the design and interpretation of experiments.

METHODS.

Cultures. Mycobacterium tuberculosis B.C.G. was grown in Sauton medium (Soltys, 1952) with 3 gm./l. B.D.H. casein hydrolysate added. To obtain dispersed cultures 0·05 p.c. Tween 80 (polyoxyethylene derivative of sorbitan monooleate) was used. Cultures grew in 10-14 days at 37° C. Surface cultures were inoculated with suspensions of the organisms in pentane and incubated 7-10 days at 37° C.

Resistant B.C.G. was obtained on the surface of Sauton medium containing 1 μg./ml. isoniazid. After slow growth on this medium the organism grew rapidly on successive transfers to media containing 5, 10 and 20 μg./ml. isoniazid. The strain was then maintained in Sauton medium to which 20 μg./ml. isoniazid was added at the time of inoculation.

Preparation of suspensions. Dispersed cultures were washed and resuspended in the presence of 0·05 p.c. Tween 80. Surface cultures were suspended by gentle grinding in a Potter-Elvehjem homogeniser or by spreading the surface growth on filter paper and working it for a few seconds with a spatula to obtain a butyrous consistency. The material was then washed and suspended in the required medium. The rate of uptake of isoniazid by dispersed and surface cultures was the same with the following suspending media: distilled water, physiological saline, 0·05 p.c. Tween 80 and McIlvaine buffers of pH 6, 7 and 8.

Cultures of other organisms. Escherichia coli and *Staphylococcus aureus* were grown on meat infusion agar for 24 hours, aerobically at 37° C. *Bacillus megaterium* was grown

on serum agar for 24 hours, aerobically at 30° C. *Mycobacterium phlei* grew on meat infusion agar in 48 hours aerobically at 37° C. All were washed in saline.

Measurement of isoniazid uptake. Suspensions of the organisms were shaken with radioactive isoniazid in Warburg manometer flasks at 130 strokes/min. under air unless otherwise stated. Suspensions contained 20-30 mg./ml. dry weight of cells and samples of 0·3-0·5 ml. were removed into 5 ml. of saline, centrifuged and washed three times more before the cells were transferred to weighed planchettes. The washings were completed within half an hour. Final cell concentrations of isoniazid were the same when saline, distilled water, 0·05 p.c. Tween 80 or solutions of unlabelled isoniazid were used as washing liquids. The stability to washing of the bound radioactivity described by Barclay *et al.* (1953a) was not found in the present experiments. Stable counts were obtained after three washings and maintained up to nine rapid washings, but cells suspended overnight in saline or distilled water lost up to 80 p.c. of their radioactivity. For this reason the cells were washed rapidly as described above.

The washed cells were dried in an oven at 100° C. or under an infra-red lamp. Radioactive samples were counted with a Philips electronic counter model PW4035. Corrections for self-absorption were applied with samples of more than 3 mg. dry weight. Radioactive isoniazid, labelled in the carbonyl group, was obtained from the Radio Chemical Centre, Amersham, England.

Radioactive pyridoxal *iso*nicotinoyl hydrazone was obtained by heating equimolar solutions of pyridoxal and radioactive isoniazid in a boiling water bath for 1 hour then evaporating to dryness. The residue was dissolved in distilled water when required. Insufficient material was available for isolation but free isoniazid was not detected with picryl chloride (Cuthbertson and Ireland, 1952).

RESULTS.

Temperature. The rate of uptake of isoniazid by viable and heat-killed cells increased with increasing temperature as shown in Table 1. The uptake of isoniazid was linear with time at 18° and 28° C. but at 38° C. curves were obtained similar to those shown in Fig. 1. The rate at 38° C. was approximately linear in the 60-minute interval used.

TABLE 1.

The effect of temperature on isoniazid uptake.

Sensitive B.C.G. from Sauton surface cultures shaken in saline with 50 μg./ml. isoniazid.

Temperature °C.	Isoniazid uptake μg. isoniazid/gm. dry weight/hr.	
	Viable cells	Dead cells
18	44	22
28	72	29
38	98	48

Concentration of isoniazid. The uptake of isoniazid in the presence of different concentrations of isoniazid is shown for viable cells in Fig. 1 and for heat-killed cells in Fig. 2. The uptake of isoniazid by heat-killed cells is contrary to the finding of Barclay *et al.* (1953a) who reported no uptake by killed cells. It is possible that this discrepancy may be explained by the higher levels of isoniazid employed in the present experiments.

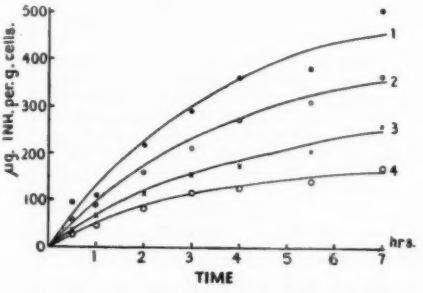

Fig. 1. *Isoniazid uptake with varying external concentrations of isoniazid.* Sensitive B.C.G. from Sauton surface cultures shaken in saline at 35° C. at 130 strokes/min. with (1) 200, (2) 100, (3) 50, and (4) 20 μg./ml. isoniazid.

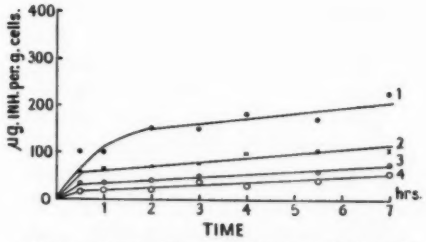

Fig. 2. *Isoniazid uptake by non-viable cells with varying external isoniazid concentrations.* Sensitive B.C.G. from Sauton surface cultures killed by boiling. Suspensions in saline shaken at 35° C. with (1) 200, (2) 100, (3) 50, and (4) 20 μg./ml. isoniazid.

Uptake of isoniazid in the presence of oxidisable substrates. The presence of oxidisable substrates such as glycerol or glucose or a full growth medium depressed the uptake of isoniazid while stimulating respiration. Fig. 3 shows this depression of isoniazid uptake. It was found that the final level of radioactivity taken up was also reduced by the presence of substrates.

Aeration. The rate of uptake of isoniazid was increased by shaking suspensions in air and when a maximum rate had been achieved, further stimulation was observed

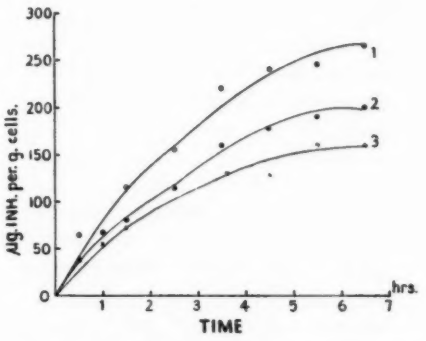

Fig. 3. *Isoniazid uptake in the presence of oxidisable substrates.* Sensitive B.C.G. from Sauton surface cultures suspended in saline and shaken at 35° C. with 60 μg./ml. isoniazid with (1) no added substrate, (2) 6 p.c. glycerol, (3) Sauton medium.

when the gas atmosphere was enriched with oxygen. Fig. 4 shows the effect of aeration on isoniazid uptake. The degree of aeration had no effect on the uptake of isoniazid by killed cells. Under anaerobic conditions living cells took up very little isoniazid. By comparison, it appeared that stationary suspensions of viable cells were virtually anaerobic and this was in keeping with the high endogenous respiratory activity found in *Mycobacteria*. Reference will be made later to the uptake of isoniazid by resistant organisms. Although the levels of isoniazid taken up by resistant B.C.G. and *E. coli* were low, they showed the same stimulating effect of aeration which has been described above.

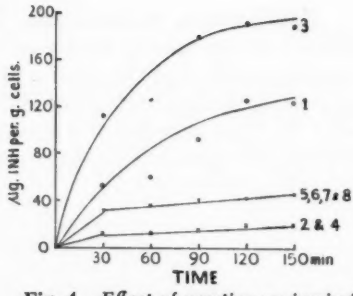

Fig. 4. *Effect of aeration on isoniazid uptake.* Sensitive B.C.G. from Sauton surface cultures suspended in saline and shaken at 35° C. with 50 µg./ml. isonazid. Numbers 1-4 viable cells: numbers 5-8 heat-killed cells. Numbers 1 and 5 shaken under air; 2 and 6 shaken under nitrogen; 3 and 7 shaken with oxygen; 4 and 8 stationary under air.

Suspensions used to measure the effect of substrates on isoniazid uptake were adjusted to a suitable density to ensure that in the experimental conditions there was always adequate aeration for the increased oxygen requirement.

From the experiments so far described, it was recognised that isoniazid was taken up in two ways. These may be described as "metabolic" and "non-metabolic". The former is increased by aeration and occurs only with viable cells. This may represent an uptake which requires energy for transfer into the cell or possibly an actual metabolic change of the isoniazid. The other process which is observed with killed cells or anaerobic viable suspensions appears to be a diffusion into the cell or some form of combination on the cell surface. This distinction was further observed in the presence of chemical inhibitors.

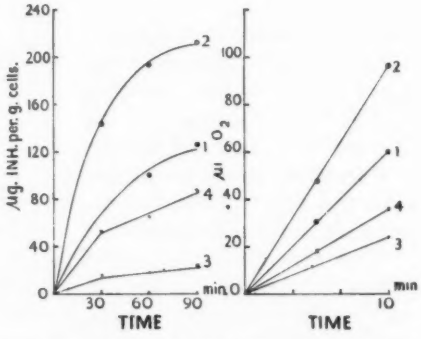

Fig. 5. *Chemical stimulation and inhibition of isoniazid uptake and the endogenous respiration.* Sensitive B.C.G. from Sauton surface cultures suspended in saline and shaken at 35° C. with 60 µg./ml. isoniazid. The endogenous respiration rates were measured during the first 30-minute interval after isoniazid was added. (1) Control, (2) 2,4-dinitrophenol $5M \times 10^{-4}$, (3) sodium cyanide $M \times 10^{-3}$, (4) potassium fluoride $M \times 10^{-2}$.

Chemical inhibitors. Fig. 5 shows the effect on respiration and isoniazid uptake of 2,4-dinitrophenol, sodium cyanide and potassium fluoride. The endogenous respiration rates were determined

during the first 30-minute interval of isoniazid uptake. 2,4-Dinitrophenol produced an initial stimulation of isoniazid uptake which was followed later by a decreasing level of radioactivity in the cells (Fig. 8). The time elapsing before the inhibition effect appeared varied with the concentration of the compound. Thus at a concentration of $5M \times 10^{-4}$ the inhibition appeared at approximately 9 hours. The residual isoniazid uptake in the presence of $M \times 10^{-3}$ sodium cyanide was the same as that observed in dead cells. Sodium azide produced complete inhibition of isoniazid uptake at $M \times 10^{-3}$ and 90 p.c. inhibition at $M \times 10^{-4}$, but at these concentrations did not inhibit endogenous respiration. These inhibitors did not affect the uptake of isoniazid by dead cells and further distinguished between the sensitive "metabolic" process and the insensitive "non-metabolic" process.

Effect of cell concentration. It will be clear that the effect of cell concentration on uptake of isoniazid is related to the oxygen requirement of the "metabolic" type of uptake. Even light suspensions may be inadequately supplied with oxygen when incubated in stationary tubes. Tables 2 and 3 show that in stationary suspensions the amount of isoniazid taken up per mg. dry weight of cells decreased with increasing cell concentration, but in shaken suspensions the uptake of isoniazid was the same for a heavy washed cell suspension as in a lighter suspension which was comparable in density to a culture.

TABLE 2.

The effect of cell concentration on the isoniazid uptake.

Sensitive B.C.G. from Sauton surface cultures suspended in saline with 20 μg./ml. isoniazid at 35° C.

Cell concentration mg./ml. dry weight	Isoniazid uptake μg./gm. dry weight		
	1 hr.	4 hr.	23 hr.
70 stationary	4	8	23
7 stationary	11	20	32
1·5 stationary	22	47	60
7 shaken at 100/min.	58	114	210

TABLE 3.

The effect of cell concentration on the isoniazid uptake.

Sensitive B.C.G. from Sauton surface cultures shaken in saline with 20 μg./ml. isoniazid at 35° C.

Cell concentration mg./ml. dry weight	Isoniazid uptake μg./gm. dry weight		
	1 hr.	2 hr.	20 hr.
20	61	100	166
2	58	88	168

Influence of pH. The rate of uptake of isoniazid was unaffected by pH in the range pH 6-8, even when the cells were exposed in the buffers for 24 hours before testing. However, a depression of isoniazid uptake occurred after several hours' exposure in flasks where no alkali was present to absorb carbon dioxide formed during respiration. This effect was more pronounced at pH 6 and could be increased by the addition of carbon dioxide to the gas atmosphere. It therefore appeared that carbon dioxide exerted some effect on isoniazid uptake which could not be attributed to a lowering of pH.

Chelating compounds. Attention has been drawn to the chelating ability of isoniazid (Cymerman-Craig, Rubbo, Willis and Edgar, 1955). Cells which had been exposed previously to *o*-phenanthroline or ethylene diamine tetra-acetate, took up isoniazid at the same rate and to the same extent as untreated cells. Cells exposed to *o*-phenanthroline were stained pink. These chelating compounds were also without ability to displace the isoniazid from the cells.

Acetone dried cells. Because preparations of cells dried with acetone or with acetone and ether are often employed by biochemists, it is worth recording that these took up isoniazid like dead cells. If, therefore, the "metabolic" uptake of isoniazid is essential to the action of isoniazid, results with acetone dried cells will be misleading.

The uptake of isoniazid by other organisms. Organisms of other genera took up comparatively little isoniazid. Fig. 6 shows the initial rate of uptake with varying external concentrations of isoniazid, by sensitive B.C.G., *Staphylococcus aureus, Escherichia coli* and *Mycobacterium phlei.* At 24 hours the pattern was unchanged except for a relative increase in uptake by *M. phlei.* In other experiments it was also found that *Proteus mirabilis, Bacillus megaterium* and *Candida albicans* took up little isoniazid. The presence of growth medium did not affect the uptake by *E. coli* and *B. megaterium. E. coli* showed a slight stimulation of uptake with aeration. A complete examination of all these organisms was precluded by the large amounts of labelled isoniazid which would have been required. None of the data obtained was inconsistent with the general conclusion that the organisms which were insensitive to isoniazid took up negligible amounts of the compound whether aerated or not and in the presence and absence of growth media.

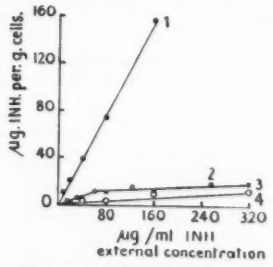

Fig. 6. *Comparison of isoniazid uptake by different genera of bacteria.* The uptake of isoniazid in the first 30 minutes at 35° C. with stationary suspensions in saline of (1) sensitive B.C.G., (2) *E. coli,* (3) *Staph. aureus,* and (4) *M. phlei* with different concentrations of isoniazid.

Uptake of isoniazid by resistant B.C.G. Two patterns of behaviour have emerged for suspensions of resistant B.C.G. These are illustrated in Figs. 7 and 8. Fig. 7 shows the uptake of isoniazid by resistant B.C.G. suspended in saline and shaken in

flasks which contained no alkali for the absorption of carbon dioxide. Fig. 8 shows the maintenance of resistance when the cells were suspended in saline, buffer or medium with alkali present in the flasks. Resistant B.C.G. grown in the presence of radioactive isoniazid did not become labelled.

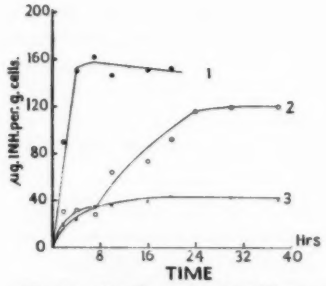

Fig. 7. *Uptake of isoniazid by sensitive and resistant B.C.G.* Sensitive B.C.G. from Sauton surface cultures and resistant B.C.G. from surface cultures on Sauton medium with 20 μg./ml. isoniazid added. Cell suspensions in saline shaken with 20 μg./ml. isoniazid at 35° C. without alkali present in Warburg flasks. (1) Sensitive B.C.G., (2) resistant B.C.G., (3) killed suspensions of sensitive and resistant B.C.G.

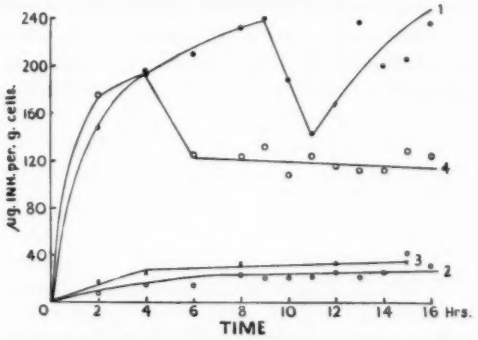

Fig. 8. *Uptake of isoniazid by sensitive and resistant B.C.G.* Sensitive B.C.G. from Sauton surface cultures and resistant B.C.G. from surface cultures on Sauton medium with 20 μg./ml. isoniazid added. Cell suspensions in phosphate buffer pH 7 shaken with alkali present in flasks, with 20 μg./ml. isoniazid. (1) Sensitive B.C.G., (2) resistant B.C.G., (3) killed sensitive and resistant B.C.G., (4) sensitive B.C.G. with 2,4-dinitrophenol $2{\cdot}5M \times 10^{-4}$.

The loss of resistance seen in Fig. 7 appeared to be due to the accumulation of carbon dioxide and this was further confirmed by the experiment shown in Table 4.

TABLE 4.

Effect of carbon dioxide on the uptake of isoniazid by resistant B.C.G.

Resistant B.C.G. from surface cultures on Sauton medium containing 20 μg./ml. isoniazid, shaken in saline with 20 μg./ml. isoniazid at 35° C. for 24 hours. Suspension (1) with added carbon dioxide, (2) without alkali to absorb carbon dioxide, (3) with alkali present, (4) non-viable cells.

Suspension	Isoniazid uptake μg./gm. dry weight
1	96
2	68
3	36
4	30

Fig. 8 also illustrates a phenomenon observed with sensitive B.C.G. which cannot at present be explained. This is the decline in the radioactive count of cells between 8 and 12 hours followed by an increase between 12 and 16 hours.

This has been repeatedly observed, although it is easily missed (see Fig. 7) when sampling times are too widely spaced. The effect was not eliminated by terramycin, which is bacteriostatic, at 100 and 50 μg./ml. Sodium azide and 2,4-dinitrophenol were also tested on account of their ability to inhibit growth, but these caused inhibition of isoniazid uptake as already described.

Pyridoxal isonicotinoyl hydrazone. Boone, Strang and Rogers (1956) reported stimulation of isoniazid uptake by pyridoxal. Table 5 shows stimulation of isoniazid uptake by different concentrations of pyridoxal. The dependence on

TABLE 5.

Effect of pyridoxal on isoniazid uptake.

Sensitive B.C.G. from dispersed Sauton medium cultures suspended in Tween 80 solution with 50 μg./ml. isoniazid at 37° C.

Concentration of pyridoxal HCl in μg./ml.	Isoniazid uptake μg./gm. dry weight		
	15 min.	30 min.	60 min.
50	35	49	62
20	29	40	51
10	23	32	43
5	22	29	40
0	16	29	40

pyridoxal concentration and the fact that isoniazid and pyridoxal were present in equimolar concentrations before any pronounced stimulation was observed, suggested that the hydrazone might be formed and taken up more rapidly than free isoniazid.

It will be seen from Table 6 that the stimulating effect of pyridoxal on isoniazid uptake was observed with living and dead cells and that the *iso*nicotinoyl

TABLE 6.

Effect of pyridoxal on isoniazid uptake.

Sensitive B.C.G. from Sauton surface culture shaken in saline at 35° C. with isoniazid (INH) 50 μg./ml., or pyridoxal *iso*nicotinoyl hydrazone (P-INH) at a concentration equivalent to 50 μg./ml. isoniazid, with pyridoxal-HCl (P) at 40 μg./ml.

	Isoniazid uptake μg./gm. dry weight				
	30 min.	60 min.	90 min.	120 min.	150 min.
Viable cells with INH	42	72	130	135	138
Viable cells with INH+P	57	108	143	157	177
Viable cells with P-INH	87	124	133	157	157
Viable cells with P-INH+P	93	93	109	149	154
Heat-killed cells with INH	21	28	32	46	40
Heat-killed cells with INH+P	37	53	65	80	92
Heat-killed cells with P-INH	123	163	173	174	179
Heat-killed cells with P-INH+P	123	192	179	192	216

hydrazone of pyridoxal was taken up rapidly by living and dead organisms. The addition of pyridoxal had no effect on the uptake of the hydrazone. These results were in keeping with the theory that the stimulating effect of pyridoxal was due to the formation of the hydrazone and further suggested, since living and dead cells took up the hydrazone readily, that the uptake of the hydrazone was by a "non-metabolic" process.

Isoniazid uptake in cultures. Barclay *et al.* (1953a, 1953b, 1954, 1955) employed *Mycobacterium tuberculosis* H37Rv in their studies. Table 7 shows that the uptake of isoniazid by cultures of B.C.G. was similar to that by cultures of H37Rv. Although the figures quoted by Barclay *et al.* (1954) were for levels after exposure for 5 days, they have reported that the uptake occurred chiefly in the first 24 hours.

TABLE 7.

Comparison of isoniazid uptake by Mycobacterium tuberculosis *H37Rv and B.C.G.*

Data for H37Rv from Barclay *et al.* (1954) with Dubos medium. Data for B.C.G. obtained with dispersed Sauton cultures exposed to isoniazid for 24 hours at 37° C.

Concentration of isoniazid μg./ml.	Isoniazid uptake μg./gm. dry weight	
	H37Rv	B.C.G.
0·4	5·4	—
0·5	—	8
1·0	—	11·5
2·0	—	18
2·95	24·3	—
5·0	—	28
5·8	36·6	—
7·0	—	38
11·3	49·1	—

DISCUSSION.

From the experiments described it was concluded that it is not necessary to look for an enzyme which is peculiar to isoniazid-sensitive *Mycobacteria* as the site of action of isoniazid. Naturally resistant organisms, such as *E. coli* and *Staph. aureus*, etc., take up little isoniazid. The behaviour of resistant B.C.G. is not fully understood, but they do not become labelled in cultural conditions. The loss of resistance which occurs on shaking saline suspensions in the absence of alkali to absorb carbon dioxide, probably indicates a change in permeability and, as isoniazid is then taken up to levels comparable with sensitive B.C.G., it is suggested that the enzymes concerned in "metabolic" uptake are present in resistant B.C.G.

It must be realised clearly that the relationship between the uptake of isoniazid and the bacteriostatic action of the drug has not yet been established.

We do not yet know whether the "metabolic" type of isoniazid represents an uptake mechanism which requires energy or whether isoniazid is itself changed in the process. Until this has been determined, it is necessary to carry out biochemical tests with cell material which has been exposed to isoniazid in conditions which permit the maximum "metabolic" uptake, that is, in adequately aerated suspensions with a time duration of 4-8 hours. Experiments with acetone-dried powders or cell extracts may be misleading. It is also necessary to determine for any enzymic reaction whether the degree of inhibition by isoniazid depends on the substrate concentration. Such a dependence was shown in the transaminase reaction where the inhibition by isoniazid was influenced by the concentration of keto acid (Youatt, in press).

It was shown that the same amount of isoniazid was taken up by a suspension of 20 mg./ml. dry weight as by one of 2 mg./ml. dry weight provided aeration was adequate. It is not therefore necessary to assume that higher levels of isoniazid need be present to inhibit enzymes in washed cell suspensions than are required to produce bacteriostasis in cultures. It must be emphasised that the oxygen consumption of washed suspensions of *Mycobacteria* is very high and care is needed to ensure that the degree of aeration is adequate.

SUMMARY.

An attempt has been made to establish a basis of comparison between the use of isoniazid in bacteriostatic tests and biochemical experiments.

Two processes of isoniazid uptake have been recognised. "Metabolic" isoniazid uptake occurs in viable cells under aerobic conditions and is susceptible to a number of chemical inhibitors. "Non-metabolic" uptake persists in killed cells, under anaerobic conditions and in cells poisoned by chemical inhibitors.

It is suggested that, until the relationship between the uptake of isoniazid and its bacteriostatic action has been established, biochemical tests should be made on cells exposed to isoniazid in conditions which permit the "metabolic" uptake.

Organisms of other genera took up little isoniazid and their resistance may therefore be due to their failure to take up the drug rather than to the absence of susceptible enzyme systems.

Resistant B.C.G. suspensions normally do not take up isoniazid, but washed suspensions exposed to carbon dioxide take up isoniazid to levels comparable with sensitive organisms. This is thought to be due to a modification of the permeability of the cells.

Chelating compounds, such as *o*-phenanthroline, neither prevented the uptake of isoniazid nor displaced the bound radioactivity.

The stimulating effect of pyridoxal on the uptake of isoniazid is considered to be due to the formation of the hydrazone. The hydrazone is taken up more

rapidly by both living and dead cells and is considered to be a "non-metabolic" process.

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THE INDUCTION OF IMMUNOLOGICAL TOLERANCE IN RATS TO FOREIGN ERYTHROCYTES

by G. J. V. NOSSAL[1]

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Since the principle of immunological tolerance was first clearly demonstrated by Medawar and his associates (Billingham *et al.*, 1953), many workers have attempted to extend the concept from the field of homograft immunity to the field of classical immunology. The most definitely established example of immunological tolerance to a classical antigen is the phenomenon of tolerance to bovine serum albumin in rabbits, induced by neonatal injections of the antigen (Hanan and Oyama, 1954; Cinader and Dubert, 1955). Claims have also been made for the induction of tolerance to micro-organismal antigens such as Trichomonas foetus antigen (Kerr and Robertson, 1954) and Salmonella pullorum (Buxton, 1954). However, experiments with a basically very similar design (Burnet, Stone and Edney, 1950; Cohn, 1957; Nossal, 1957b) failed completely to demonstrate immunological tolerance.

Woodruff and Simpson (1955) showed that in rats, the period during which tolerance to the tissues of another rat could be obtained extended to about fourteen days of life. In view of this, it was thought that the rat might prove specially suitable for the study of tolerance problems; if Woodruff's finding can be applied to other immune systems, the need for operative intra-uterine foetal injections disappears. Furthermore, a paper by Simonsen (1955) suggested that some degree of tolerance to human erythrocytes could be obtained by inoculating chick embryos 2-3 days before hatching; however, the degree of tolerance was greatly enhanced by giving repeated injections of human erythrocytes in the first few weeks of life. The plan of the present experiments was therefore to inject day-old rats with washed erythrocytes and to continue the injections at twice-weekly intervals for varying periods of time. Evidence is presented that such procedures lead to true immunological tolerance of prolonged duration; that this is not merely immune paralysis; and that the tolerance is species but not strain specific.

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[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra, A.C.T., Australia.

Materials and Methods.

Rats. Wistar rats 3-6 months old were used for breeding; except where stated, infant rats were injected within the first 36 hours of life, and usually within the first 6 hours. Rats were weaned at four weeks of age, and fed on a standard diet of cubes and tap water *ad libitum*. All rats were individually earmarked. Blood samples were obtained from the tail artery and veins, and serum prepared in the conventional manner.

Antigens. Erythrocytes from various animal species were used as the antigens throughout this series of experiments. The donor animals were: two humans, G.N. (Blood group O, Rh positive), and J.G. (Blood group A, Rh positive), one guinea pig, two sheep and numerous inbred C3H mice. Blood from donor animals was stored in an equal volume of Alsever's solution containing 50 units per ml. of penicillin and streptomycin. The cells were kept for up to three weeks at 4° C. On the days that the cells were to be injected, they were centrifuged at 500 × *g.* for 15 minutes, resuspended in physiological saline, respun, resuspended and finally respun at the same speed. From this sediment of washed packed cells, the final antigen was prepared, were it 20 p.c. cells in physiological saline, 2 p.c. cells, etc. Also, the cells used in the antibody titrations were prepared in the same way.

Antibody titrations. Serum samples were routinely inactivated at 56° C. for 30 mins. and titrated on the day of preparation. The antibody content of the serum was determined by making serial twofold dilutions of serum in physiological saline, in plastic trays containing multiple cups. Then an equal volume (0·25 ml.) of 1 p.c. erythrocytes in saline was added. The patterns of agglutination were read at 1-2 hrs., at room temperature, depending on the species of animal whose cells were used. The titre of the serum was expressed as the reciprocal of the initial dilution of serum giving a standard pattern of partial agglutination.

In some of the preliminary experiments, the haemolysin titre of the serum was determined as well. This was done by incubating the serum dilutions and 1 p.c. red cells at 37° C. for 30 mins., and then adding an equal volume of 12 units of guinea-pig complement, and incubating for a further 30 mins. In the case of the mouse and guinea-pig red cells, the haemolysin titre was much lower than the haemagglutinin titre. With sheep cells, the titre was considerably higher. Animals tolerant by one titration technique were always tolerant by the other, and therefore the haemolysin technique was considered unnecessary for the bulk of the experiments.

EXPERIMENTAL.

Establishment of the phenomenon.

The first series of experiments was designed to show the immunological effect of injecting 0·25 ml. of 20 p.c. erythrocytes on the day of birth, and subsequently twice per week for either three or eight weeks. All animals were bled at nine weeks, and then challenged with an injection of 1 ml. of 20 p.c. cells. They were then bled one week later, at ten weeks, together with normal control rats of the same age. Control rats in all the experiments were subjected to a preliminary bleeding, but on no occasion had demonstrable agglutinin titres. At eleven weeks, the rats were given a further injection of 1 ml. of 20 p.c. cells, and finally bled again at twelve weeks. In Figs. 1-5 the subscript "0" refers to the bleeding at nine weeks, subscript "1" refers to that at 10 weeks, and the subscript "2" to the bleeding at twelve weeks. The subscripts "3 wk" and "8 wk" refer to the duration of the neonatal injection course. The subscript "CONT"

refers to control animals. This convention is used in almost all the diagrams. Fig. 1 refers to injection with C3H mouse cells, 2 to injection with sheep cells, 3 and 4 to injection with human "J.G." and "G.N." cells, and 5 to injection with guinea-pig cells. The results indicate clearly that an eight-week post-natal injection course leads quite uniformly to immunological non-reactivity in the treated animals. The results after a three-week post-natal course are considerably more variable; on the whole there is a tendency for "partial tolerance" to be conferred. In this series of experiments, after an eight-week injection course, tolerance was complete for mouse and sheep erythrocytes and somewhat less for human and guinea-pig cells.

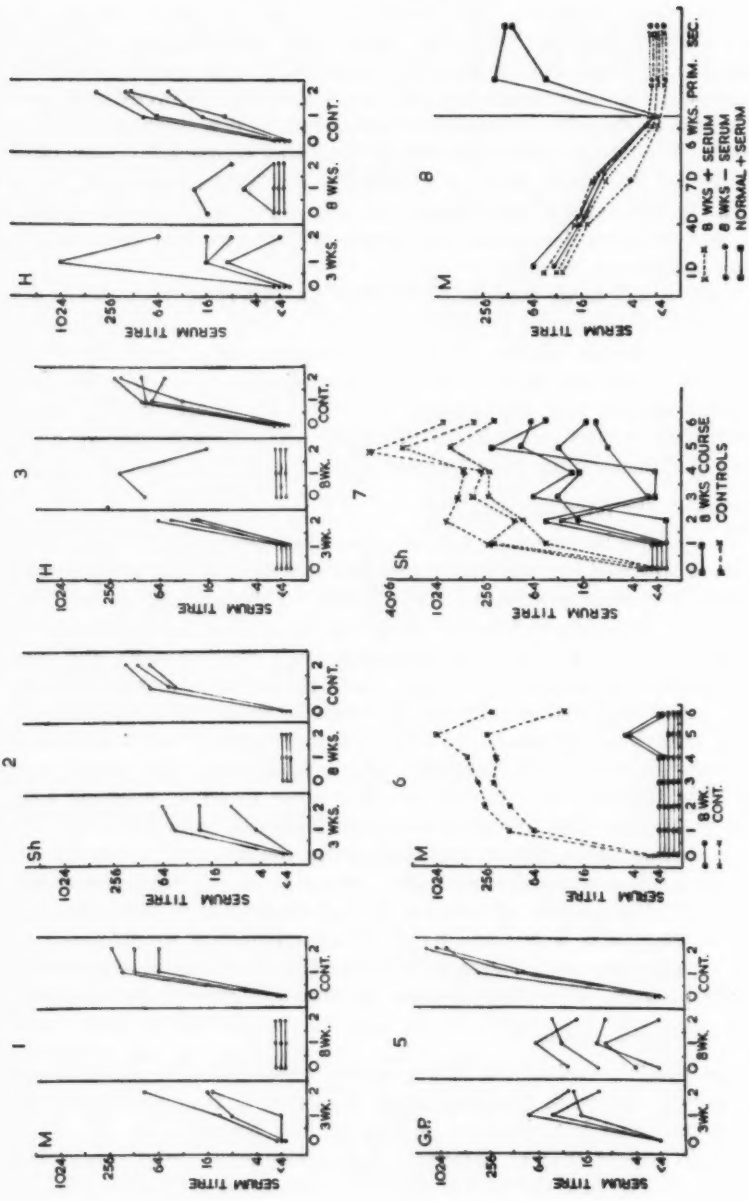
Next, it had to be established whether the observed phenomenon was true immunological tolerance, or merely a form of immune paralysis, such as was described by Felton (1949). In order to determine this, three lines of approach were used. First, the duration of the observed non-reactivity in the absence of antigenic stimuli was determined; secondly, the effect of the injection of hyperimmune serum into tolerant rats was measured; and thirdly, attempts to induce true immune paralysis by very large doses of antigen were made.

Duration of the phenomenon.

Two of the litters which had an eight-week neonatal injection course have now been observed for 9 months. One litter had injections of sheep erythrocytes, the other of mouse erythrocytes. The history of the animals up until 12 weeks is similar to those quoted in the foregoing experiment, namely, a preliminary bleeding, and then primary and secondary challenge injections, and bleedings. Subsequently the animals were rested until they were 4 months old, and then had a further injection of 1 ml. of 20 p.c. cells, the tertiary injection, followed a week later by a bleeding, and after a further rest of one week, they had a quarternary injection and bleeding. The animals were then rested until they were eight months old. At this age they were given at intervals again two weeks apart, their fifth and sixth challenge injections, with bleedings one week after each injection. Figs. 6 and 7 illustrate the results respectively of injection with C3H mouse and sheep cells. It can be clearly seen that at all stages of their life, the animals exhibited a marked depression of the normal immune response, compared with controls. There is a tendency for tolerance to wear off in experiments using sheep cells, but using mouse cells, the tolerance remained complete. These findings were confirmed by several similar experiments.

Effect of immune serum.

One possible explanation of our results is that the observed immunological non-reactivity is due to the persistence of antigen in the tissues, and that antibody is produced but absorbed as soon as it is formed. On this hypothesis, one would expect that an injection of hyperimmune serum would have the following effects: first, the titre of passively transferred antibody in the recipient animal should fall rather sharply, due to *in vivo* reaction with antigen; secondly,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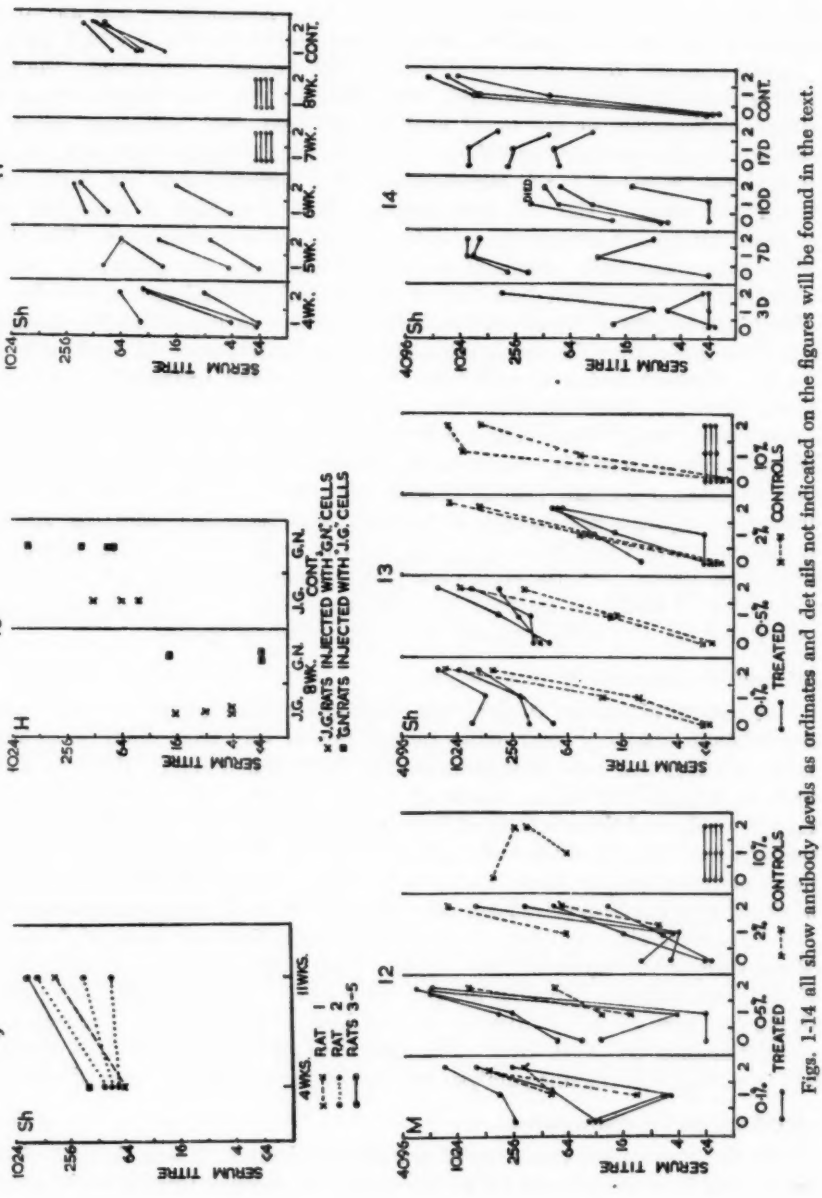

Figs. 1-14 all show antibody levels as ordinates and det ails not indicated on the figures will be found in the text.

after the passively transferred antibody had been metabolized, and all excess antigen neutralized, the animal should then be capable of reacting with a normal immune response to an injection of the relevant antigen. Accordingly, the following experiment was performed. One litter of rats was made tolerant to mouse erythrocytes in the usual way by an eight-week post-natal injection course. At nine weeks of age, three rats in the litter, together with two normal control rats received 1 ml. intravenously of a hyperimmune rat anti-mouse erythrocyte serum. This had been prepared from a number of adult rats immunized by several intraperitoneal injections of mouse erythrocytes. The titre of the serum was 1600. Three of the tolerant rats were left uninjected. Serial blood samples were taken from the rats which had received serum, in order to determine the rate of decay of transferred antibody in normal and in tolerant rats. Six weeks after the serum injection, when the rats were 15 weeks old, all the rats received an injection of 1 ml. of 20 p.c. mouse erythrocytes, and two weeks later a further injection. One week after each of these injections the animals were bled. The results are illustrated in Fig. 8. It is evident that the rate of decay of antibody, shown on the left-hand side of the diagram, is identical in tolerant animals and in controls. Furthermore, six weeks later, the animals were just as non-reactive to "primary" and "secondary" antigenic stimuli as the animals that had received no serum. This is illustrated on the right-hand side of the figure. It is therefore clear that the non-reactivity is not due to the persistence of antigenic material in a site accessible to the circulation. This experiment has also been repeated twice with identical results.

Attempts to induce immune paralysis.

It seemed of interest to show whether more mature animals could be rendered immunologically non-reactive by injection of very large doses of antigen. Such an effect had been demonstrated by Dixon and Maurer (1955) in their work on tolerance to bovine serum albumin in rabbits. Accordingly, two rats aged 8 weeks and three rats aged 3 weeks were taken and injected in the following way. The first 8-week-old rat (Rat 1 in Fig. 9) received 3 ml. of 50 p.c. sheep erythrocytes five times per week for three weeks, and the second eight-week-old rat (Rat 2) received one injection only of 15 ml. of packed sheep cells. The three-week-old rats (Rats 3-5) received 5 injections per week of 1 ml. of 50 p.c. sheep erythrocytes for three weeks. Four weeks after the commencement of each immunization schedule the rats were bled. Six weeks after this again, all the rats were given a further injection of 1 ml. of 20 p.c. cells. The response was determined one week later. The results are given in Fig. 9. It is obvious that in no case was immune paralysis induced by these excessive doses of antigen.

Specificity of the phenomenon.

In the view of the specificity of homograft tolerance, it was thought of interest to see how specific was the tolerance induced towards foreign erythrocytes. Accordingly, the two groups of rats made tolerant to the human cells of G.N.

and J.G. (respectively O, Rh positive and A, Rh positive) which have already been cited in Figs. 3 and 4 were given an injection of 1 ml. of the opposite cells and bled one week later. The titrations were also performed with the opposite cells, i.e., those tolerant to G.N. cells were injected with, and titrated against, J.G. cells, and vice versa. The results are given in Fig. 10. They indicate that the animals given an 8-week course, after challenge with the opposite cells, have titres ranging from < 4 to 20 (left-hand side of Fig. 10). The control rats (right-hand side of the figure) have titres ranging from 40 to 800. Thus the animals remain relatively tolerant even though injected with cells of a different blood group.

Similarly, a litter of rats tolerant to C3H mouse cells were given an injection of 1 ml. of 20 p.c. C57B mouse cells. They were still completely tolerant to the latter cells when bled one week later. The animals described in Fig. 5 which had been made tolerant to guinea-pig cells, were injected with 1 ml. of 20 p.c. sheep cells, and one week later were bled and the serum titrated for anti-sheep agglutinins. The results showed that a normal immune response ensued. From these results, it can be seen that the tolerance induced by the neonatal injection course is species specific, but not strain specific.

Relationship of length of injection course to degree of tolerance achieved.

It has been observed that in animals injected for three weeks after birth there was a tendency for partial tolerance to be induced. However, there was considerable variation in this, both from animal to animal and from experiment to experiment. It was therefore important to determine how long a course of injections was needed to induce uniform tolerance. Accordingly, groups of rats were injected for 4, 5, 6, 7 and 8 weeks with 1 ml. of 20 p.c. sheep erythrocytes. At nine weeks of age they were bled, together with normal controls, and then given the usual primary and secondary injections of 1 ml. of 20 p.c. cells, two weeks apart. The results are given in Fig. 11. It is shown that in order to induce complete tolerance with regularity the injections must continue for at least seven weeks, although several animals in the other groups show marked depression of response.

Effect of varying the dose of antigen given during the tolerance course.

It is a well-established fact that doses of erythrocytes 10 or even 100 times smaller than those given in the above experiments are highly antigenic in normal animals. The following experiment was designed to determine the smallest dose of antigen which could induce tolerance. Groups of 4 rats were injected with 0·1, 0·5, 2 and 10 p.c. sheep or mouse erythrocytes, in the usual volume of 0·25 ml. twice weekly for eight weeks. At nine weeks, the animals were bled and given a "primary" stimulus of 1 ml. of the same dilution of cells as they had been receiving, and the response determined one week later. At eleven weeks, the animals all received a "secondary" injection of 1 ml. of 20 p.c. cells.

They were bled one week later. The results of these injections are demonstrated in Figs. 12 and 13 illustrating the results with C3H mouse and sheep erythrocytes respectively. From these it can be seen that the very small doses of red cells lead to immunization rather than tolerance and that uniform and complete tolerance is achieved only in the groups injected with 10 p.c. cells.

The latest time at which a tolerance injection course can be started.

Woodruff and Simpson (1955) showed that some degree of tolerance to homografts could be achieved by cell injections given to suckling rats as late as two weeks of age. Accordingly, groups of rats were inoculated for the first time at 3, 7, 10 and 17 days of life with 0·25 ml. of 20 p.c. sheep erythrocytes. They were given similar injections twice per week for eight weeks. One week later they were given their "primary" challenge, and two weeks later their "secondary" challenge of 1 ml. of 20 p.c. cells. The responses were tested as usual one week after each injection. The results, illustrated in Fig. 14, show considerable variation from animal to animal; in the group injected for the first time at 3 days, 2 out of 3 animals showed marked tolerance, and in each of the groups injected first at 7 and 10 days, one animal in the group showed distinct tolerance. In the 17-day group, the peak titres were below those of the control rats, but no true tolerance was demonstrated. Thus it appears the animals must be less than three days old for uniform tolerance to be achieved, but odd animals can be made tolerant when injected for the first time at 10 days. These findings correlate well with Woodruff and Simpson's findings in the field of homograft immunity.

DISCUSSION.

The finding of immunological tolerance was predicted by Burnet and Fenner (1949) on theoretical grounds. They postulated that antigens artificially or naturally introduced into the tissues of the developing embryo would be dealt with in the same general fashion as effete body cells, and other expendable material, without antibody formation. If the same antigen is subsequently reinjected into the same animal when it is more mature, the animal mistakes the antigen for "self" and fails to produce antibody. Since the introduction of the Burnet and Fenner "self-marker" hypothesis, the phenomenon of immunological tolerance has become an experimental fact. Furthermore, since then, two main alternative theories of antibody formation have appeared, namely Jerne's (1955) natural selection hypothesis, and Burnet's clonal selection hypothesis (1957). Each of these explain tolerance in somewhat different ways, but all theories are equally impressed with the importance of the phenomenon to immunological theory. It is therefore of the utmost importance to determine under what general circumstances tolerance can or cannot be induced. The literature on this question is still somewhat confused, but work done in this

laboratory has allowed several concepts to emerge more clearly. Extensive experiments with chicks, mice and rats have shown (Burnet, Stone and Edney, 1951; Nossal, 1957b) that it is impossible to induce tolerance to an agent such as influenza virus. Using a very similar experimental design, however, the present experiments show that tolerance can be readily induced to cellular antigens, such as washed erythrocytes. It appears, therefore, that the response to such antigens injected early in life is qualitatively different.

The present experiments show that large doses of antigen must be presented to the tissues of the developing animals for long periods of time in order to induce tolerance. The large dose necessary may be partly due to the fact that, on any theory of antibody formation, the antigen in order to produce tolerance must reach every cell potentially capable of reacting to it, whereas in an ordinary immune response, the participation of only a few cells, such as those of the regional lymph node, is sufficient to induce antibody formation. In this connection, be it noted that the injections were given by the intraperitoneal route; presumably this would result in poorer dissemination than intravenous inoculations. The necessity of the presence of the antigen in the tissues for long periods is also in line with known facts. In the classical homograft experiments, live potentially replicating cells are injected into the embryo or neonate. These presumably survive as long as the tolerance persists, and lead to the presence of the relevant antigens in the host's tissues for long periods. Our results could be due to the fact that each cell must be exposed to the antigen for a long time before it and its descendants are rendered tolerant; alternatively, different cell lines may go through the critical period during which tolerance can be induced at different times of the animal's life. It would then be necessary to continue the injections till all the cells potentially capable of antibody formation had gone through this critical period. Thirdly, it may simply require repeated opportunities before all the cells which are potential antibody producers, or the ancestors of potential antibody producers, are effectively modified by the antigen.

The whole concept of immunological tolerance has been attacked by Bauer *et al.* (1956) who failed to induce tolerance to sheep erythrocytes in rats following a single injection. They postulate that the whole range of phenomena included under the heading of tolerance may all be examples of immune paralysis, "the immaturity of the animal acting as a facilitating factor". It is difficult to reconcile this point of view with our results. The known examples of immune paralysis have been clearly shown to be due to the persistence of antigens in the tissues for long periods of time (Stark, 1955; Dixon and Maurer, 1955). It seems rather unlikely that erythrocyte surface antigens could persist for many months in the tissues of a rat, particularly after the injection of an excess of antibody. Moreover, it is impossible to produce even transient non-reactivity in more mature animals with much greater doses of antigen.

The author feels that the system described above has many advantages for the study of tolerance problems. Admittedly it is tedious to have to inject animals for many weeks. However, once the animals are rendered tolerant, they remain so uniformly and almost completely for many months. Moreover, there is no need for elaborate operations, and the titration technique is both simple and accurate.

A preliminary account of this work has been published (Nossal, 1957a).

SUMMARY.

It is shown that immunological tolerance in rats to foreign erythrocytes can be induced by injecting large doses of the antigens into suckling rats on the day of birth, and continuing the injections for several weeks thereafter.

It is shown that this tolerance is of many months duration, and is not due to the persistence of antigens in a site accessible to the circulation. True immune paralysis to these antigens cannot be produced, even by excessive doses.

The phenomenon is shown to be species specific, but not strain specific.

The rats must be less than 3 days old when the injections are commenced, and the injections must continue for at least 7 weeks for full tolerance. The minimum effective dose is 0·25 ml. of 10 p.c. cells per injection.

Acknowledgments. The author wishes to express his sincere thanks to Sir Macfarlane Burnet for his interest in this work.

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THE EFFECT OF THE ISOMERS OF CHLORAMPHENICOL ON GROWTH AND INDOLE SYNTHESIS BY *ESCHERICHIA COLI* 7-4

by BARBARA McDOUGALL AND F. GIBSON

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(*Accepted for publication 3rd December, 1957.*)

Washed cell suspensions of a mutant strain of *Escherichia coli* (7-4) unable to convert indole to tryptophan were found by Gibson, Jones and Teltscher (1956a) to accumulate indole when incubated with glucose and ammonium ions. The biosynthesis of indole was later found to be strongly inhibited by bacteriostatic concentrations of chloramphenicol and the tetracyclines (Gibson, McDougall, Jones and Teltscher, 1956b).

The chloramphenicol molecule contains two asymetric carbon atoms and the four isomeric forms (D-*threo*, L-*threo*, D-*erythro* and L-*erythro*) have been isolated. Maxwell and Nickel (1954) found that D-*threo* chloramphenicol, the naturally occurring isomer, is the only active antibacterial, the others inhibiting the growth of *E. coli* only at relatively high concentrations.

This paper describes experiments comparing the activities of the four isomers in inhibiting growth and indole synthesis by *E. coli* 7-4.

MATERIALS AND METHODS.

Organism Used.

E. coli 7-4 is a mutant obtained after ultra-violet irradiation and penicillin selection techniques and has been described previously (Gibson, *et al.*, 1956a).

Media.

Heart-infusion agar. The preparation of this medium used for growing cells for suspensions has been described elsewhere by Gibson, *et al.* (1956a).

Synthetic medium. The chemically defined medium described by Davis and Mingioli (1950) was used for bacteriostatic tests. The medium was prepared double strength, preserved by the addition of a little carbon tetrachloride-toluene mixture, and diluted as required. To support the growth of *E. coli* 7-4 it was supplemented with $10^{-4}M$ DL-tryptophan. Both the glucose and tryptophan were added to the medium before autoclaving at 10 lb./in.^{2} for 10 minutes.

Preparation of Washed Cell Suspensions.

Cells for suspensions were grown on heart-infusion agar plates which were inoculated

from a heart-infusion agar slope incubated at 37° C. for 16 hr. A light suspension in sterile distilled water (0·2 ml. of approx. 4×10^6 cells/ml.) of cells from this culture was spread over each plate. The plates were incubated at 37° C. for 16 hr. and the growth washed off with 10 ml. saline (0·9 p.c.) per plate. The cells were washed twice with equal volumes of saline and resuspended in 0·2 *M* phosphate buffer pH 7·7 (Na_2HPO_4-KH_2PO_4). The turbidity was adjusted until it corresponded to a dry weight of approx. 1·5 mg./ml.

Indole Synthesis.

The washed suspension of cells in 0·2 *M* phosphate buffer was added to an equal volume of the following substrates dissolved in water:

glucose $2 \times 10^{-1}M$
NH_4Cl $10^{-2}M$
$MgSO_4$ $2 \times 10^{-4}M$

The mixture of substrates and cells was incubated in a water bath at 37° C., sampled at intervals and the indole formed was estimated as described below.

In order to eliminate any inhibition of indole formation occurring as a result of depressed formation of adaptive enzymes (Gibson, *et al.*, 1956b) the synthesis system was allowed to attain a maximum rate of indole production before chloramphenicol was added. The time required to reach a constant rate was about 4 hr. The suspension was then divided into separate portions and the appropriate concentration of antibiotic added to all except the control. Incubation and sampling were continued, usually for another 2 hr.

Indole Assay.

Indole was estimated by a colorimetric method described by Gibson, *et al.* (1956a). Briefly, this method consisted of extracting indole from an 0·5 ml. sample of the suspension with toluene and forming a coloured compound with *p*-dimethylaminobenzaldehyde under acid conditions. The colour intensity was measured in an E.E.L. portable colorimeter using a 626 filter and the corresponding concentration of indole was read from a standard curve. The results are expressed as mμ mol. indole formed per mg. dry weight of cells.

Bacteriostatic Tests.

These were set up as follows: A series of tubes (6 × ¾ in.) containing 2·5 ml. double strength synthetic medium and the appropriate volume of water was sterilized by autoclaving 10 lb./in.^{2} for 10 min. An aqueous solution of chloramphenicol was pasteurised at 80° C. for 10 min., dilutions made aseptically and the required volume added to the tubes. The tubes were then inoculated with cells taken from the plates used for preparation of cell suspensions. One drop of a light suspension (approx. 2×10^6 cells/ml.) in sterile distilled water was added to each tube. The tubes were incubated at 37° C. for 24 hr. and the turbidities measured in a portable E.E.L. colorimeter using a 626 filter. The results are expressed as the percentage inhibition of growth compared with the control containing no antibiotic.

RESULTS.

D-threo-*Chloramphenicol.*

The D-*threo* isomer is the natural active-form of chloramphenicol and was included in most experiments. The effect of this isomer on indole synthesis is shown in Figs. 1 and 3. The addition of 5 μg./ml. to a suspension producing indole at a steady rate caused almost complete inhibition of synthesis. The same concentration inhibited growth by 94 p.c. (Table 1).

TABLE 1.

Effect of chloramphenicol isomers on growth.

Concentration (μg./ml.)	Inhibition of Growth (p.c.)					
	D-*threo*	L-*threo*		D-*erythro*		L-*erythro*
5	94					
10	100			3		8
100		5	11	9	6	72
200				17		
250		28	26		21	
500		90	100		44	
1000						98

L-threo-*Chloramphenicol.*

The effect of three different concentrations of this isomer on indole synthesis is shown in Fig. 1 and the bacteriostatic effect of the same concentrations is recorded in Table 1. A concentration of 100 μg./ml. caused only 5 p.c. inhibition of growth and indole synthesis was slightly depressed. Higher concentrations caused increased inhibition of growth and 500 μg./ml., the highest concentration tested, inhibited growth by 90 p.c. This concentration depressed indole production to about the same extent as 5 μg./ml. D-*threo*-chloramphenicol. The other concentration tested, 250 μg./ml., was intermediate in its effect both with respect to indole synthesis and growth.

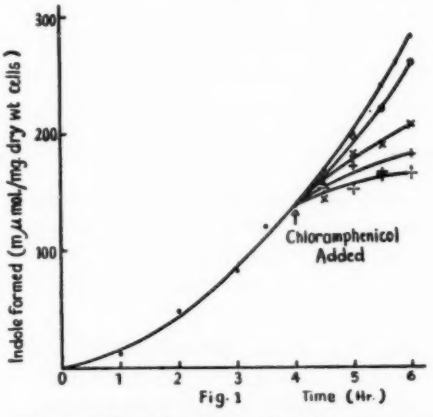

Fig. 1. Effect of L-*threo* and D-*threo* isomers of chloramphenicol on indole synthesis by *E. coli* 7-4. The cell suspension was incubated at 37° C. for 4 hr., divided into separate portions, and chloramphenicol added to give the final concentrations shown—

—●—●— No addition.
—+—+— 5 μg./ml. D-*threo*-chloramphenicol.
—○—○— 100 μg./ml. L-*threo*-chloramphenicol.
—×—×— 250 μg./ml. L-*threo*-chloramphenicol.
—+—+— 500 μg./ml. L-*threo*-chloramphenicol.

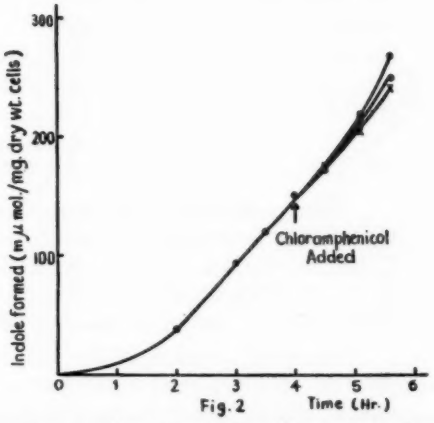

Fig. 2. Effect of D-*erythro*-chloramphenicol on indole synthesis by *E. coli* 7-4. Conditions as for Fig. 1.

—●—●— No addition.
—○—○— 100 μg./ml. D-*erythro*-chloramphenicol.
—×—×— 500 μg./ml. D-*erythro*-chloramphenicol.

D-erythro-*Chloramphenicol.*

The results of an experiment on indole formation are shown in Fig. 2 and the corresponding effect on growth in Table 1. This isomer also was bacteriostatic at higher concentrations, as shown in Table 1, but was not as active as the L-*threo* form. Thus, a concentration of 500 μg./ml. inhibited growth by 44 p.c. Indole production was not significantly inhibited by concentrations of D-*erythro*-chloramphenicol up to 500 μg./ml. (Fig. 2).

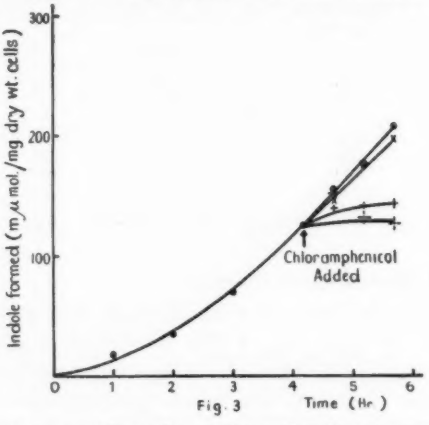

Fig. 3. Effect of L-*erythro* and D-*threo* isomers of chloramphenicol on indole synthesis by *E. coli* 7-4. Conditions as for Fig. 1.

—●—●— No addition.
—+—+— 5 μg./ml. D-*threo*-chloramphenicol.
—×—×— 100 μg./ml. L-*erythro*-chloramphenicol.
—ǂ—ǂ— 1000 μg./ml. L-*erythro*-chloramphenicol.

L-erythro-*Chloramphenicol.*

The bacteriostatic effect of this isomer is also recorded in Table 1 and it showed greater antibacterial activity than the D-*erythro* or L-*threo* forms. A concentration of 10 μg./ml., which inhibited growth by 8 p.c., had no effect on indole synthesis (Fig. 3) and 100 μg./ml. caused 72 p.c. inhibition of growth, but indole synthesis was only slightly depressed (Fig. 3). A concentration of 1,000 μg./ml., however, caused complete inhibition of both growth and indole synthesis.

DISCUSSION.

The interference of D-*threo*-chloramphenicol with aromatic biosynthesis in *E. coli* may be at least one factor in its mode of action (Gibson, *et al.*, 1956b) or the reaction inhibited may be similar to other vital reactions in the cell. The effect on indole synthesis of the other isomers of chloramphenicol which have little antibiotic activity is therefore of interest.

The small amounts of the unnatural isomers available did not permit the detailed examination of concentration-activity curves. However, the results obtained indicate that these isomers have no effect on indole synthesis at concentrations of the same order as the bacteriostatic concentration of D-*threo*-chloramphenicol (3 μg./ml.).

These findings provide support for the suggestion that the observed inhibition of indole synthesis is in some way related to the antibacterial activity of chloramphenicol.

Higher concentrations of the unnatural isomers were bacteriostatic and growth was almost completely inhibited by concentrations of the order of 500-1,000 μg. chloramphenicol/ml. The higher concentrations also depressed indole synthesis. It is of interest that the growth of the cells was apparently more sensitive to the action of the D-*erythro* and L-*erythro* isomers than was indole formation. The possibility that the cell suspensions degraded these isomers was not investigated.

The results presented are in accord with the suggestion that studies on the inhibition of aromatic synthesis by chloramphenicol will be of value in determining the mode of action of the antibiotic.

SUMMARY.

The effects of the unnatural isomers of chloramphenicol, which are relatively inactive as antibacterials, on indole synthesis from glucose and ammonium ions by washed suspensions, and growth in a chemically defined medium, are described.

Indole synthesis was almost completely inhibited by bacteriostatic concentrations (3-4 μg./ml.) of the naturally occurring (D-*threo*) chloramphenicol. At the same concentrations, the L-*threo*, D-*erythro* and L-*erythro* isomers did not inhibit indole synthesis. Much higher concentrations of these isomers were bacteriostatic and also depressed indole formation.

Acknowledgments. We are indebted to Dr. R. E. Maxwell of Parke, Davis & Company for gifts of the isomers of chloramphenicol, and to the National Health and Medical Research Council for a grant which partly supported this investigation. We wish to thank Professor S. D. Rubbo for his encouragement of this work, and Mr. G. Morrison for technical assistance.

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THE ANALEPTIC ACTIVITY OF BEMEGRIDE

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The ability of Bemegride[2] (I) (β-methyl-β-ethylglutarimide, Megimide, NP13) to antagonize, in mice, moderate hypnosis effected by several structurally related hypnotics (barbiturates, thiobarbiturates, glutarimides, diketopiperidines, diketopyridines, diketothiazanes, diketothiazolidines and monoureides) and structurally unrelated hypnotics (saturated and unsaturated alcohols, aldehydes, carbamates, cyclic ethers and sterols) has been reported previously (Shaw, Simon, Cass, Shulman, Anstee and Nelson, 1954; Shulman, 1956; Somers, 1956; Frey, Hushahn and Soehring, 1956; Kimura and Richards, 1957; and Shulman and Laycock, 1957).

This paper reports the effectiveness of Bemegride analepsis in mice to another group of structurally diverse hypnotics, namely, 5:5-diethyl-2:4-diketothiazolidine[1] (II), β-methyl-β-*n*.amylglutarimide[2] (III) (see Somers, 1956), α-methyl-α-phenylglutarimide[3] (IV), α-methyl-α-phenylsuccinimide[5] (V), N-methyl-α-methyl-α-phenylsuccinimide[3] (VI), α-ethyl-α-phenylsuccinimide[5] (VII), β-chlorvinyl-ethyl-ethynylcarbinol[4] (Placidyl) (VIII) (see Kimura and Richards, 1957) and phenyl-diethylacetamide[3] (IX).

EXPERIMENTAL.

Mice of mixed strain and sex weighing between 20 and 30 gm. were divided by random selection into test groups of 20. The analeptic activity of Bemegride was indicated by its ability to reduce the sleeping time of mice given a dose of hypnotic so chosen as to give a mean sleeping time of 60-80 minutes. The sleeping time was taken as the interval elapsing between the loss and the maintained recovery of the righting reflex. All drugs were administered intraperitoneally, Bemegride in physiological saline solution and the hypnotics in a 1 p.c. tragacanth suspension. Bemegride (20 mg./Kg.) or saline was administered at 15-minute intervals.

Each hypnotic was tested in duplicate experiments and a "t" test applied in every case.

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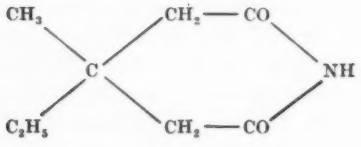
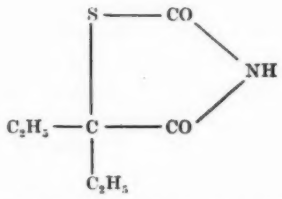
[1] F. Hoffman-La Roche & Co. A.G. (Basel). [2] Nicholas Pty. Ltd. (Australia).
[3] British Schering Research Laboratories (England). [4] Abbott Laboratories, Illinois (U.S.A.).
[5] Parke, Davis & Co., Michigan (U.S.A.).

RESULTS.

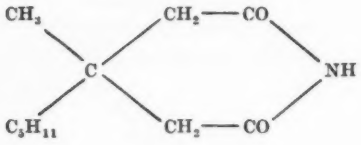
The analeptic activity of Bemegride (20 mg./Kg. at 15-minute intervals) to hypnosis induced in mice by 5:5-diethyl-2:4-diketothiazolidine (275 mg./Kg), β-methyl-β-*n*.amylglutarimide (350 mg./Kg.), α-methyl-α-phenylglutarimide (150 mg./Kg.), α-methyl-α-phenylsuccinimide (210 mg./Kg.), N-methyl-α-methyl-α-phenylsuccinimide (225 mg./Kg.), α-ethyl-α-phenylsuccinimide (150 mg./Kg.), β-chlorvinyl-ethyl-ethynylcarbinol (Placidyl) (175 mg./Kg.) and phenyl-diethylacetamide (110 mg./Kg.) is recorded in Tables 1 and 2. At the dose level of Bemegride employed there were no signs of toxicity except in the case of β-methyl-β-*n*.amylglutarimide where mild and transient convulsive episodes were observed in some mice, particularly when these animals were stimulated.



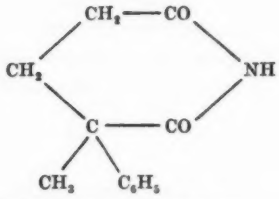
I. Bemegride
(β-methyl-β-ethylglutarimide)



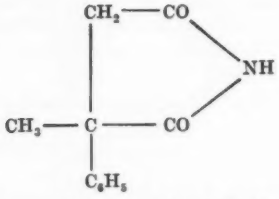
II. 5:5-diethyl-2:4-diketothiazolidine



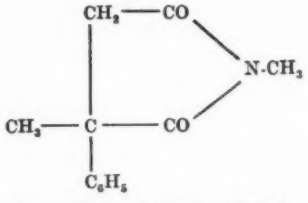
III. β-methyl-β-*n*. amylglutarimide



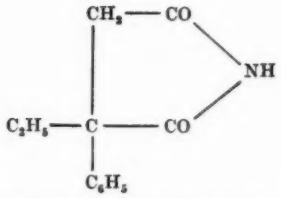
IV. α-methyl-α-phenylglutarimide



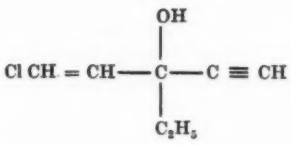
V. α-methyl-α-phenylsuccinimide



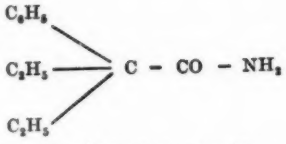
VI. N-methyl-α-methyl-α-phenylsuccinimide



VII. α-ethyl-α-phenylsuccinimide



VIII. Placidyl
(β-chlorvinyl-ethyl-ethynylcarbinol)



IX. Phenyl-diethylacetamide

TABLE 1.

	α-methyl-α-phenyl-succinimide 210 mg./Kg.		N-methyl-α-methyl-α-phenyl-succinimide 225 mg./Kg.		α-ethyl-α-phenly-succinimide 150 mg./Kg.		α-methyl-α-phenyl-glutarimide 150 mg./Kg.	
Saline Treatment	+	−	+	−	+	−	+	−
Bemegride	−	20 mg./Kg.	−	20 mg./Kg.	−	20 mg./Kg.	−	20 mg./Kg.
No. of animals taken	20	20	20	20	20	20	20	20
No. of survivors used in calculations	20	20	20	20	20	20	19	20
Mean sleeping time (min.)	68·6	15·2	63·4	13·1	77·4	23·6	60·2	8·6
Standard error (SE)	3·93		8·69		6·09		5·80	
"t" Test vs. Saline	13·58***		5·79***		8·84***		8·90***	

*** = highly significant ($p < 0·001$)

TABLE 2.

	β-methyl-β-n.amyl-glutarimide 350 mg./Kg.		5:5-diethyl-2:4-diketothiazolidine 275 mg./Kg.		Placidyl 175 mg./Kg.		Phenyl-diethyl-acetamide 110 mg./Kg.	
Saline Treatment	+	−	+	−	+	−	+	−
Bemegride	−	20 mg./Kg.	−	20 mg./Kg.	−	20 mg./Kg.	−	20 mg./Kg.
No. of animals taken	20	20	20	20	20	20	20	20
No. of survivors used in calculations	20	20	20	20	20	20	19	20
Mean sleeping time (min.)	77·3	46·6	63·2	19·0	72·3	18·8	74·6	13·9
Standard error (SE)	3·49		6·00		6·26		4·28	
"t" Test vs. Saline	8·77***		7·37***		8·55***		14·2***	

*** = highly significant ($p < 0·001$).

DISCUSSION.

The demonstration of widespread and safe analeptic activity of Bemegride to a large variety of structurally related and unrelated hypnotics should give this substance an important place both as a therapeutic and diagnostic agent in medical practice (reviewed by Shaw, 1957) and also as a pharmacological tool for the further investigation of the mechanisms subserving hypnotic, stimulant and analeptic activity.

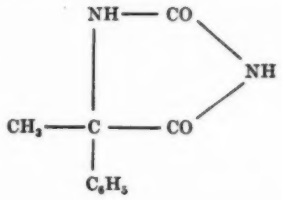
Of particular interest is the observation arising from this and prior work (Somers, 1956; Frey *et al.*, 1956; Shulman and Laycock, 1957) that Bemegride, itself a β,β-disubstituted glutarimide, antagonizes moderate hypnosis induced in mice by glutarimides disubstituted either on the α-carbon atom (α-methyl-α-phenylglutarimide and α-ethyl-α-phenylglutarimide (Doriden)) or on the β-carbon atom (β-methyl-β-(n.butyl, n.amyl or n.hexylglutarimide)). Subsequent work (Shulman and Laycock, in press) has indicated that Bemegride exhibits less pronounced antagonism, often associated with signs of toxicity, to deep hypnosis (sleeping time 4-7 hrs., minimal response to pain, marked depression of corneal reflex and frequent cyanosis) induced by both types of glutarimides. There was a high incidence of death in the β-methyl-β-n.amylglutarimide treated groups.

SUMMARY.

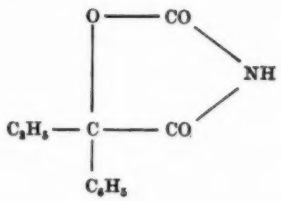
The analeptic activity of Bemegride to moderate hypnosis induced in mice by 5:5-diethyl-2:4-diketothiazolidine, β-methyl-β-*n*.amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide, N-methyl-α-methyl-α-phenylsuccinimide, α-ethyl-α-phenylsuccinimide, β-chlorvinyl-ethyl-ethynylcarbinol (Placidyl) and phenyl-diethylacetamide has been indicated. Particular reference has been made to antagonism of hypnosis induced by both α-α and β-β-disubstituted glutarimides.

ADDENDUM.

Bemegride (20 mg./Kg. at 15-minute intervals) has been further shown to antagonise ($p \leqslant 0{\cdot}001$) moderate hypnosis in mice (sleeping time 60-80 minutes) induced by α-methyl-α-phenylhydantoin (220 mg./Kg.) (X) (Parke, Davis & Co., U.S.A.) and α-ethyl-α-phenyl-2:4-diketooxazolidine (225 mg./Kg.) (XI) (Mallinckrodt Chemical Works, U.S.A.).



X. α-methyl-α-phenylhydantoin



XI. α-ethyl-α-phenyl-2:4-diketooxazolidine

Acknowledgments. We wish to thank Abbott Laboratories, Illinois, U.S.A., British Schering Research Laboratories Ltd. (England), F. Hoffman-La Roche & Co. A.G. Basel, Mallinchrodt Chemical Works, U.S.A., Nicholas Pty. Ltd. (Australia), and Parke, Davis & Co., Michigan, U.S.A., for their generous gifts of drugs used in this investigation.

We are indebted to Mr. K. Healy for technical assistance.

We are most grateful to Dr. K. H. Pausacker for his helpful criticism and to Professor R. D. Wright for his advice and guidance during this investigation.

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PRIMARY ISOLATION OF INFLUENZA A VIRUS: THE OPTIMAL CONDITIONS FOR AMNIOTIC INFECTION[1]

by E. L. FRENCH AND J. K. DINEEN

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(*Accepted for publication 27th January, 1958.*)

Some years ago investigations carried out by Beveridge and Burnet (1946) indicated that 13- to 14-day-old embryos were superior to 10- to 12-day-old embryos for the isolation of influenza virus. Since then this Institute has consistently used 13- or 14-day-old embryos for isolation of influenza virus by the amniotic route. American workers have recently used 10-day-old chick embryos to isolate influenza virus from throat washings (Mogabgab *et al.*, 1956; Takemoto *et al.*, 1955), and the second edition of "Diagnostic Procedures for Virus and Rickettsial diseases" (1956) recommends the use of 10-day embryos for this purpose.

The influenza epidemic in Melbourne during the winter of 1956 provided a group of throat washings, known to contain influenza virus, for the re-investigation of this question. A series of experiments were undertaken, the results of which indicate that the isolation of this strain of influenza virus was more readily achieved in 13- to 15-day-old embryos than in younger embryos.

MATERIALS AND METHODS.

Collection of throat washings. Within 48 hours of the onset of symptoms patients gargled 10-15 ml. of physiological saline and about 3 ml. of sterile nutrient broth were added. Portions of the throat washings were sealed in hard glass ampoules and rapidly frozen in alcohol-dry ice mixtures and then transferred to the dry ice cabinet for storage.

Inoculation of chick embryos. Fertile eggs from white leghorn or Australorp flocks were incubated before inoculation at 39° C. and after inoculation at 35° C. The embryos were inoculated using a minor modification of the method of Taylor and Chialvo (1942).

Complement fixation. The technique of Donnelley (1951) with the modification of 2 drops per volume instead of 1 drop was used.

Virus haemagglutinin. Virus haemagglutinin was determined by the pattern test with 1 p.c. fowl or human group O (IV) erythrocytes which were allowed to settle at room temperature (20-22° C.).

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[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra, A.C.T., Australia.

EXPERIMENTAL.

An ampoule of throat washing was removed from the dry ice cabinet and placed in a 37° C. water bath until only a small sliver of ice was still present. If necessary, the contents of the ampoules were centrifuged to remove large particles and a small quantity of concentrated penicillin and streptomycin solution added to give approximately 500 units of each per ml. Dilutions of the throat washings were made in 10 p.c. horse serum saline containing 500 units each of penicillin and streptomycin per ml. The dilutions of throat washing were inoculated amniotically into 11-day, 13-day and 15-day embryos (0·05 ml. per embryo) with an 0·25 ml. syringe graduated in 0·01 ml. The inoculations were made as rapidly as possible by two people working together. While awaiting inoculation the dilutions were kept at 4° C. and the time elapsing between

TABLE 1.

Throat washing "Tynan".

Age of embryo at inoculation	No.	Number of days after inoculation							
		3 days				5 days			
		dilution of throat washing				dilution of throat washing			
		10^{-0}		10^{-1}		10^{-0}		10^{-1}	
		Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract
11 day	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—
	5	—	—	—	—	—	—	—	—
	6	—	—	—	—	—	—	—	—
13 day	1	—	4000/480	—	—	—	—	—	—
	2	—	—	—	—	200/10	—	—	—
	3	—	—	—	—	—	—	—	—
	4	—	—	—	—	160/20	—	—	—
	5	—	—	—	—	1000/40	—	—	—
	6	—	40/—	—	—	—	—	—	—
15 day	1	—	—	—	—				
	2	—	—	—	—				
	3	—	—	—	—				
	4	—	2560/160	—	—				
	5	D	D	—	—				
	6	D	D	D	D				

4000/480 = the titre obtained using Human cells/Fowl cells.
D = embryo dead. No haemagglutinin detected.
— = no haemagglutinin detected with either type of cells.
0 = not available for examination.

removal of the throat washing from the dry ice cabinet and its inoculation into chick embryos did not exceed 60 minutes. Twelve 11- and 13-day embryos and six 15-day embryos were inoculated with each dilution of throat washing.

After three days' incubation at 35° C. six embryos of each age and from each dilution were removed from the incubator and the amniotic fluids and lungs collected individually. After a further two days' incubation the remaining embryos were harvested in a similar manner. Each pair of lungs was ground with alundum and taken up in 1 ml. of saline. Amniotic fluid and lung extracts were titrated for haemagglutinin with 1 p.c. human and 1 p.c. fowl erythrocytes.

Three different throat washings (Tynan, Reddy and Parsons), known to contain infective influenza virus, were tested in the manner described. Table 1 shows the results with a throat washing "Tynan" which contained only a small amount of virus. "Reddy" throat washing contained more virus and yielded some positive results in the 10^{-1} dilution (Table 2). "Parsons" throat washing contained detectable virus when diluted 1 in 100 (Table 3).

It is quite obvious from these Tables that 11-day-old embryos are inferior to the older embryos. If only 11-day-old embryos had been used with throat

TABLE 2.

Throat washing "Reddy".

Age of embryo at inoculation	No.	Number of days after inoculation							
		3 days				5 days			
		dilution of throat washing				dilution of throat washing			
		10^{-0}		10^{-1}		10^{-0}		10^{-1}	
		Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract
11 day	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	3	—	—	—	—	D	D	D	D
	4	—	—	—	—	0	D	D	D
	5	D	D	D	D	D	D	D	D
	6	D	D	D	D	D	D	D	D
13 day	1	—	—	30/10	30/15	—	—	320/40	800/160
	2	—	—	160/40	20/5	—	—	—	—
	3	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—
	5	—	—	120/30	40/5	D	D	—	—
	6	20/20	1000/250	—	—	D	D	1000/160	320/60
15 day	1	120/80	640/160	—	—				
	2	10/5	1000/180	160/80	750/200				
	3	30/10	320/50	80/10	320/60				
	4	20/5	640/80	240/40	960/320				
	5	—	—	—	—				
	6	40/20	1280/100	—	—				

TABLE 3.

Throat washing "Parsons".

Age of embryo at inoculation	No.	Number of days after inoculation											
		3 days						5 days					
		dilution of throat washing						dilution of throat washing					
		10^{-0}		10^{-1}		10^{-2}		10^{-0}		10^{-1}		10^{-2}	
		Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract	Amniotic fluid	Lung extract
11 day	1	40/15	320/10	—	—	—	—	—	—	160/20	—	—	—
	2	200/15	320/10	10/—	400/80	30/—	1000/200	10/—	400/80	320/40	—	—	—
	3	80/20	15/10	—	—	—	—	—	—	10/—	10/—	—	—
	4	—	100/—	80/5	0	—	—	80/5	0	80/40	160/20	—	—
	5	20/10	320/80	40/—	350/100	—	—	40/—	350/100	—	—	D	D
	6	50/—	400/100	D	D	—	—	D	D	400/100	100/15	D	D
13 day	1	80/15	600/200	40/20	1600/800	—	—	—	15/—	160/15	—	10/—	—
	2	80/5	320/60	—	—	80/10	1600/150	0	—	320/20	10/—	40/—	10/—
	3	160/5	320/80	500/150	1600/600	—	700/50	0	—	—	—	—	—
	4	80/15	400/80	40/—	400/100	—	—	—	10/—	100/20	—	320/40	—
	5	—	40/—	—	80/—	—	—	0	10/—	80/5	200/20	80/10	320/20
	6	320/20	800/300	D	D	120/—	4800/400	D	D	D	D	D	D
15 day	1	320/40	1600/400	—	—	—	—						
	2	700/300	600/200	400/80	1600/300	70/—	1200/350						
	3	500/150	1200/200	300/150	4000/1000	—	—						
	4	320/40	2000/400	80/15	3200/800	—	—						
	5	400/100	2000/600	400/80	3200/800	—	—						
	6	—	—	100/20	1600/400	35/—	1200/350						

washings "Tynan" and "Reddy" no virus would have been detected. There is little difference between the sensitivity of 13-day-old and 15-day-old embryos harvested at 3 days. The 13-day embryos have the advantage of being available for 5 days' incubation after inoculation whereas 3 to 4 days is the maximum remaining time for incubation of 15-day embryos.

The 15-day embryos have one important advantage: they are more resistant to bacterial infection and trauma than 13-day or 11-day embryos. On several occasions tracheal scrapings heavily infected with antibiotic resistant staphylococci were inoculated into the amniotic cavity of 15-day embryos. After 3 days' incubation the embryos were still alive although the lungs and amniotic fluid contained bacteria, as well as influenza virus. The greater part of the bacteria were removed by centrifugation of the lung extract, the supernatant was then passed diluted 1/100 to 1/1000 with antibiotic to another series of 15-day embryos. The lungs removed from this second series were free from bacteria and contained influenza virus in high concentration.

After 3 days' incubation haemagglutinin was detectable in lung at high titre. The haemagglutinin titres obtained with lung suspensions and amniotic fluids infected with dilutions of "Parsons" throat washings were reduced to a more convenient form by taking the arithmetic average of the power transformations as given in Table 4. The graphical representation of these data (see Fig. 1) emphasizes that:

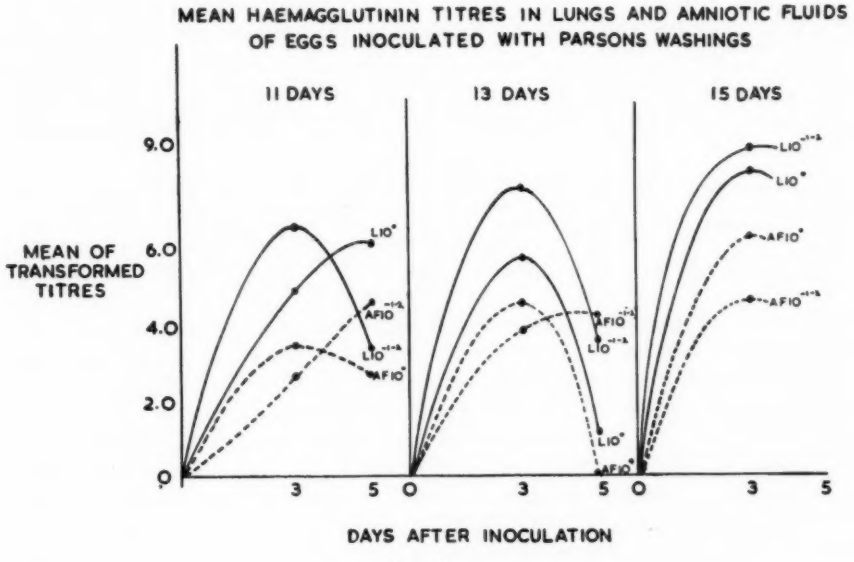

Fig. 1.

1. Higher haemagglutinin titres were detected in lungs than in amniotic fluids.
2. Higher titres were in 15-day embryos than 13-day embryos than 11-day embryos.
3. Generally the titres were higher at 3 days than at 5 days; a sharp drop in the haemagglutinin titre was frequently observed at 5 days. Consequently, these results show that embryos inoculated at 15 days and the lungs harvested 3 days later provide the best material for the isolation of influenza A strains.

We were surprised to find titres with human cells ranging up to 4000 in the lung extracts harvested after 3 days from the older embryos. This high titre of haemagglutinin in the chick embryo lung suspension suggested that this material might be suitable for determining the type of influenza virus present. Using immune ferret sera and human red cells, haemagglutinin inhibition tests with first passage lung suspension gave essentially similar results to those obtained using allantoic fluid adapted virus and fowl cells. The chick embryo lung emulsion was then absorbed three times at 4° C. with packed human cells to remove haemagglutinin and the resultant material was found to be a suitable source of the type specific complement fixing antigen. In this way the type of influenza virus could be determined within 3 days of inoculating the throat washings.

Recently one of us (E.L.F.) had an opportunity of applying this rapid method of isolation and identification to a practical problem. Through the courtesy of Dr. K. A. Lim of the Bacteriology Department of the University of Malaya we received two specimens of throat washings ("SIV" and "PAR") from cases of influenza in Singapore on May 19, 1957. These were immediately inoculated amniotically into 13-day-old chick embroys. Three days later on May

TABLE 4.

Complement fixation reactions with soluble antigens from chick embryos lungs inoculated with infected throat washings.

Antigens diluted 1:5	Anti A serum 5	10	20	40	80	160	Serum Control	Anti B serum 5	10	20	40	80	160	Serum Control
Standard A	4	4	4	4	2	—	—	—	—	—	—	—	—	—
Standard B	—	—	—	—	—	—		4	4	3	1	—	—	
"PAR"	4	4	4	4	2	1		—	—	—	—	—	—	
"SIV"	4	4	4	4	1	—		—	—	—	—	—	—	
Normal chick lung.	—	—	—	—	—	—		—	—	—	—	—	—	

4 = complete fixation of $3HD_{50}$ of complement
3 = 75 p.c. fixation of $3HD_{50}$ of complement
2 = 50 p.c. fixation of $3HD_{50}$ of complement
1 = 25 p.c. fixation of $3HD_{50}$ of complement
— = NO fixation of $3HD_{50}$ of complement

22 the lungs and amniotic fluid were harvested from these embryos and found to contain haemagglutinin for human and fowl cells. The amniotic fluid was titred and used in antihaemagglutinin tests with standard sera which were found not to inhibit haemagglutination by the isolated viruses. The lungs were extracted in saline and then absorbed three times with human red cells as described above. This material was then used in complement fixation tests with human sera known to contain type A and type B complement fixing antibody against the soluble antigens. Table 4 shows the results of this test.

TABLE 5.

Means of transformed titres of haemagglutinin in chick embryo lungs infected with "Parsons" throat washing.

Age of embryo at inoculation	Number of days after inoculation							
	3 days				5 days			
	10^0		Pooled 10^{-1}	Pooled 10^{-2}	10^0		Pooled 10^{-1}	Pooled 10^{-2}
	Amniotic fluid	Lung	Amniotic fluid	Lung	Amniotic fluid	Lung	Amnitoic fluid	Lung
11 day	3·5	5·0	2·6	6·7	2·7	6·2	4·5	3·4
13 day	4·6	5·9	4·0	7·5	0	1·2	4·2	3·3
15 day	6·4	8·1	4·8	8·7	—	—	—	—

The data have been reduced by taking arithmetic means of the titres transformed according to the expression $y = \log_{10} \frac{x}{5} / \log_{10} 2$ where y = transformed value and x = original titre.

DISCUSSION.

The experiments described here indicate that 13- to 15-day-old chick embryos were more sensitive to virus in throat washings and tracheal swabs than 11-day-old chick embryos. This finding is in agreement with that published previously by Beveridge and Burnet (1946).

Burnet (1940) studied the histological result of infection of the amniotic cavity of 12-day-old chick embryos. He showed that the result of infection is first a desquamation of the epithelium of the parabronchi which are embryologically growing and expanding rapidly at this stage of development, followed by an almost complete disorganisation of the architecture of the embryo lung. The very high haemagglutinin titres found in extracts of the infected chick embryo lungs 3 days after inoculation, led to the conclusion that the virus was multiplying in the lungs. Watson and Coons (1954) using the ingenious technique of staining with fluorescent antibody produced evidence that in their

experiments both influenza A and B types multiplied mainly in the cells lining the amnion and epidermal and pharyngeal epithelium. However, their embryos were 7 to 11 days old when inoculated and at this stage of development the pharyngeal plug is still intact and the amniotic fluid excluded from the lungs. Watson and Coons admit that when 12-day embryos were used for inoculation some of the embryos showed evidence of involvement of the cells lining the respiratory tract. The pharyngeal plug in chick embryos disappears round about the twelth day of incubation, and this may account for the differences noted by Watson and Coons in the embryos of the two age groups.

The use of 13- to 15-day-old embryos for the primary isolation of influenza virus allows not only a greater number of isolations to be made, but identification of the isolated virus by antihaemagglutinin tests and complement fixation tests with the type specific soluble antigen can be made within three days of obtaining the throat washings. Hummeler, Kravis and Sigel (1952) have also described a rapid method for identification of newly isolated influenza viruses. These workers used extracts of amniotic and chorioallantoic membranes from embryos that had been inoculated intra-amniotically with throat washings when 11 days old and harvested 48 to 72 hours later. Using this material they obtained typing of influenza virus in some cases with the primary inoculated embryos. The high titre of haemagglutinin for human cells and the presence of considerable amounts of soluble antigen in infected chick embryo lungs obtained in the present investigation, allows detection of the type of influenza virus and the antigenic subtype to be determined using human cells in antihaemagglutination reactions with standard sera.

The optimum conditions for the isolation and typing of influenza virus type A from throat washings are essentially as recommended by Beveridge and Burnet (1946). The throat washings should be inoculated intra-amniotically into chick embryos not less than 13 days old and the present study indicates that 15-day embryos are more effective than 13-day embryos. The lungs and amniotic fluid should be harvested 3 to 5 days later according to the age of the embryo when inoculated and the amniotic fluid and lung emulsion tested for haemagglutinin both with fowl and human erythrocytes. If passage is undertaken both amniotic fluid and lung emulsion should be passed amniotically to embryos 13 to 15 days old. With *post mortem* or material heavily contaminated with bacteria, 15-day-old embryos are more resistant to bacterial growth than younger embryos and one or two passes of lung suspension at 3-day intervals in the older embryos frequently isolates the virus and frees it of bacteria (Taft and French, 1957).

SUMMARY.

Thirteen- to fifteen-day-old chick embryos are more sensitive than eleven-day-old embryos for the primary isolation of influenza virus.

The presence of type specific soluble complement fixing antigen and of haemagglutinin for human red cells in the primary infected chick embryo lungs enables the serological type and subtype of the isolated virus to be determined rapidly.

Using this technique the 1956 A prime strains and A-Asian-1957 strains of virus isolated in Melbourne were typed within three days of receiving the throat washings.

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THE IMMUNOLOGICAL RELATIONS OF THE CONTAGIOUS PUSTULAR DERMATITIS VIRUS TO THE MAMMALIAN POX GROUP

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(*Accepted for publication 28th January, 1958.*)

Contagious pustular dermatitis (C.P.D.) virus causes a pustular eruption in sheep and goats and occasionally in man. The lesions produced are confined to the epithelial tissue and are similar to those produced by pox viruses in their natural hosts.

The relationships of the C.P.D. virus have been in doubt for many years, for although the similarity in size and brick-shaped form (Abdussalam, 1953) and the relationship between C.P.D. and goat pox (Bennet *et al.*, 1944) suggest a similarity with the pox group, the evidence is not sufficient to warrant inclusion in the group. Downie and Dumbell (1956) place the virus near the pox group, but will not include it within the group.

On the other hand, the virus will not adapt to growth on the chorioallantoic membrane of the chick embryo, and Lloyd *et al.* (1951) and Berger (1955) tentatively exclude the virus from the pox group on the grounds that they could not show immunological cross reactions between C.P.D. and vaccinia.

Preliminary investigations on the morphology of the C.P.D. virus and on its ability to adapt to the chick embryo confirmed the above-mentioned reports. Electron micrographs of the virus showed brick-shaped bodies with rounded ends. Internally, some of the elementary bodies showed an electron dense central area which was round in appearance and electron dense areas which occurred at the ends of the elementary body. The dimensions as calculated from the measurement of electron micrographs were 270 mμ in length and 160 mμ in width.

Several attempts were made to adapt the C.P.D. virus to the chorioallantoic membrane of the chick embryo by alternate passage in the susceptible sheep but without continued success. The virus would cause distinct lesions on the chorioallantoic membrane up to the fifth alternate passage, but the number of lesions slowly decreased and by the sixth alternate passage no virus could be detected. Serial passage of the virus on the chorioallantoic membrane was carried out after each alternate passage. The first generation membranes showed

small pinpoint lesions covering the whole membrane; the maximum size was 0·5 mm. in diameter. On the second serial passage the number of lesions decreased and by the fourth serial passage no lesions could be detected. Histological examination of the first passage membranes showed localised areas of necrosis, proliferation of the three layers and a large number of giant cells. Cytoplasmic inclusion bodies were detected in a small proportion of cells of the ectoderm when stained by Laidlaw's method.

Preliminary work was also carried out on the growth of the C.P.D. virus in tissue culture as a source of virus for immunological studies. The virus will multiply in embryonic sheep skin tissue culture, but is not cytopathogenic for monolayer cultures. In one experiment the titre of the C.P.D. virus increased from an I.D. 50 of 1·3 (log. units) in the first passage to an I.D. 50 of 4·6 in the third passage. The virus was grown in suspended sheep skin fragments and titrated by intradermal inoculation in the groin of a susceptible sheep. Growth of the virus in the sheep was, however, adopted, as large quantities of virus could be obtained from one animal.

It is the purpose of this report to show antigenic similarity and immunological cross reactions between C.P.D., vaccinia and ectromelia viruses.

Materials and Methods.

Viruses.

(1) The strain of C.P.D. used for immunisation of sheep in New Zealand;
(2) The Gillard strain of vaccinia;
(3) The Moscow strain of ectromelia virus;
(4) The strain of fowl pox virus used in New Zealand for immunisation of birds.

Antigens.

C.P.D. antigen. This antigen was prepared from fourth day vesicles of an infected sheep. The non-wool-bearing area on the inside of the thigh was cleaned with water and alcohol and the virus suspension scarified in with a piece of 40-mesh wire gauze held in artery forceps. By the fourth day vesicles appeared along the scarification marks. The surface of the vesicles was rinsed with 70 p.c. alcohol and moistened with dilute buffer solution, pH 7·2 (buffer solution was prepared according to McIlwaine's tables and diluted 1/50 in normal saline). The vesicles were gently scraped with a blunt scalpel and the vesicular fluid collected in buffered saline. The suspension obtained was shaken with glass balls and centrifuged at 300 r.p.m. for 5 minutes. Floating fatty material and wool were removed with a swab and discarded. The opalescent fluid was stored at $-20°$ C. as stock virus suspension.

Preparation of elementary body suspensions of C.P.D. Relatively pure elementary body suspensions were prepared by differential centrifugation, following the system used by Hoagland, Smadel and Rivers (1940) for preparing elementary body suspensions of vaccinia. The supernatants and deposits were saved during the differential centrifugation for immunological investigation and were designated as follows:

First angle centrifuge supernatant.
First angle centrifuge deposit.
Second angle centrifuge supernatant.
Third angle centrifuge supernatant.
Deposit at 40,000 r.p.m. for 1 hour.
Supernatant at 40,000 r.p.m. for 1 hour.

Vaccinia and fowl pox antigens. These antigens were prepared on the chorioallantoic membrane of the fertile hen's egg. Eggs previously incubated for 12 days were inoculated with a dilution of the virus so as to give confluent lesions when harvested after a 3-day incubation period. The harvested membranes were shaken in dilute phosphate buffer pH 7·2 with glass balls; 2 ml. of buffered saline were added per membrane. The tissue was deposited by low speed centrifugation and the supernatant spun in an angle head centrifuge at 3,000 r.p.m. for 1 hour to deposit the virus. The supernatant fluid was saved as the soluble antigen and the deposit as virus suspension.

Ectromelia antigen. This antigen was prepared from the livers of infected mice by the method described for vaccinia and fowl pox antigens.

Antisera.

C.P.D. antiserum was prepared in the sheep, vaccinia in the rabbit and ectromelia in the mouse by infection of the animals and bleeding 2 weeks later. Sheep serum inactivated at 56° C. for 30 minutes was found to be anticomplementary and this was overcome by inactivation at 56° C. for 45 minutes. Rabbit and mouse sera were inactivated at 56° C. for 30 minutes and were not complementary.

Techniques of tests.

Complement fixation test. Complement fixation tests were carried out using twofold dilutions of serum against twofold dilutions of stock C.P.D. antigen. Guinea pig serum stored at − 25° C. was used as complement and was titrated to a 50 p.c. haemolysis end point before use. 4 M.H.D. of complement (four 50 p.c. haemolytic doses) in the unit volume of 0·1 ml. were used throughout except in the serum and antigen control tubes which received 2,4 and 8 M.H.D. All dilutions were made in calcium magnesium saline. Complement was added to antigen dilutions before serum and the mixture was kept overnight at 4° C. and at room temperature for one hour before adding 0·2 ml. of sensitised cells. The sensitised cells were prepared by mixing equal volumes of haemolysin containing 5 M.H.D. and 3 p.c. washed sheep cells; this mixture was allowed to stand at room temperature for at least 15 minutes before addition to the overnight mixture. The racks were placed in a waterbath at 37° C. for 30 minutes and after removal the tubes were centrifuged at 2,000 r.p.m. for 2 minutes. The last tube showing 50 p.c. haemolysis or less was taken as the end point.

Double diffusion tests. Double diffusion tests were carried out in 100 mm. by 8 mm. test tubes as described by Gispen (1955). The antiserum set in sterile agar at the bottom of the tube is separated from the antigen by a neutral agar column. The antigens diffuse into the central agar column against the antibodies diffusing from below and zones of precipitation appear in the neutral agar column. The results were read one and two weeks after preparation.

The precipitable fractions were numbered according to Gispen (1955), Roman numerals being used to denote major fractions, and Arabic numerals for minor fractions.

Differential double diffusion test. If two antigens were precipitated by the same antiserum, a mixture of equal parts of both antigens, each in double amounts, was allowed to diffuse against this serum. If a duplication of precipitation zones occurred it was assumed that the antigens differed in some respect. If intensification of precipitation zones occurred it was assumed that the antigens were similar.

Cross neutralisation tests. Cross neutralisation tests between C.P.D., vaccinia and ectromelia viruses were carried out in various test animals. C.P.D. virus against C.P.D., vaccinia and ectromelia antisera in the sheep and rabbit by intradermal inoculation, ectromelia virus against the 3 antisera in the mouse by intradermal inoculation and vaccinia virus against the 3 antisera on the chorioallantoic membrane of the chick embryo.

RESULTS.

In this study advantage was taken of the availability of virus antigens as purified elementary bodies and as supernatants obtained during purification to investigate the nature of the soluble antigens mentioned briefly by Abdussalam (1953).

Complement fixing antigen.

Complement fixation tests carried out with sheep antiserum on the various deposits and supernatants obtained during purification of the virus showed that the antigen is soluble in buffered saline at pH 7·2. The results are shown in Table 1. It will be seen that most of the antigen went into solution very readily

TABLE 1.

Complement fixation titres of fractions obtained during purification of the C.P.D. virus.

Fraction	C.F. Titre
First angle centrifuge supernatant	1:2048
First angle centrifuge deposit	1:4
Second angle centrifuge supernatant	1:4
Third angle centrifuge supernatant	1:0
Deposit at 40,000 r.p.m. for 1 hour	1:16
Supernatant at 40,000 r.p.m. for 1 hour	1:128

giving a complement fixing titre of 1:2048, while the deposit gave a C.F. titre of only 1:4. Some of the soluble antigen was retained by the virus particle and was liberated slowly, for the supernatant after centrifugation of the 3,000 r.p.m. deposit at 40,000 r.p.m. for one hour in a model "L" spinco ultracentrifuge showed a titre of 1:128. Ultracentrifugation of the first angle centrifuge supernatant at 40,000 r.p.m. would not deposit the complement fixing antigen.

The soluble C.F. antigen was investigated for heat stability or lability by heating small quantities to either 70° C. or 90° C. for one hour. Checker board type C.F. tests carried out on the heated and unheated antigens showed no difference in titres and showed that the antigen was completely heat stable.

Cross complement fixation titres obtained with C.P.D. antiserum against pox group antigens.

Cross complement fixation tests carried out between C.P.D. antiserum and the mammalian pox group antigens gave a certain amount of cross fixation. The fixation occurred only with serum at a dilution of 1:4, but such fixation occurred with antigen dilutions down to 1:256. The results are shown in Table 2. The antigen titre with the homologous antiserum was 1:4096. Reciprocal tests carried out between vaccinia antiserum and C.P.D. virus gave identical results with those recorded in the Table. No cross fixation occurred between C.P.D. antiserum and fowl pox antigen.

TABLE 2.

Cross complement fixation and double diffusion tests between C.P.D. and pox group antigens.

Test	Serum	Antigens			
		C.P.D.	Ectromelia	Vaccinia	Fowl Pox
Complement fixation (*a*) Serum titre (*b*) Antigen titre	C.P.D.	1:64 1:4096	1:4 1:256	1:4 1:256	0 0
Double diffusion	C.P.D. Vaccinia	1′, 2′ III (2′)	2′ (III) III	2′ (III) 1, 2, III IV	0 0

Double diffusion tests.

In the double diffusion experiments vaccinia and ectromelia showed a common precipitation zone with C.P.D. The zone corresponded with zone III of vaccinia or ectromelia against vaccinia antiserum. In each case the zone of precipitation was of minor intensity and was denoted with an Arabic numeral followed by a dash (Table 2). No zones of precipitation occurred when the fowl pox antigen was allowed to diffuse against C.P.D. or vaccinia antiserum.

The virus particles themselves play no part in forming the precipitation zones for, if thoroughly washed pox group virus antigens were allowed to diffuse against their homologous antiserum, no zones of precipitation occurred.

The differential diffusion tests carried out between vaccinia or ectromelia and C.P.D. virus and C.P.D. antiserum gave a single zone of precipitation which corresponded with zone III of vaccinia. Intensification of the zone occurred suggesting that there is a common antigenic factor present in these 3 viruses.

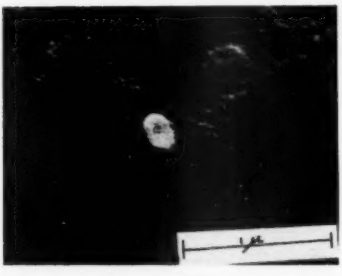

Fig. 1. Electron micrograph of the virus of contagious pustular dermatitis.

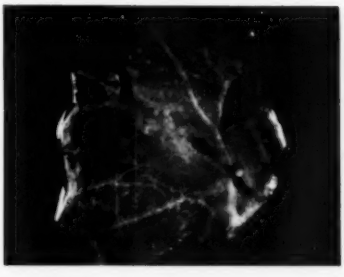

Fig. 2. First generation membrane infected with the virus of contagious pustular dermatitis; three days after infection of 12-day-old chick embryos.

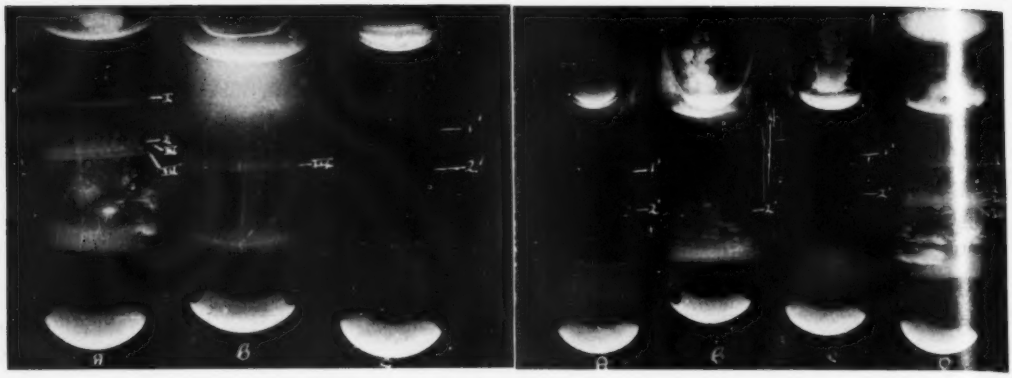

Fig. 3. Double diffusion patterns of contagious pustular dermatitis virus and pox group antigens.

Fig. 3a. Diffusion patterns of vaccinia, ectromelia and C.P.D.

A. Vaccinia antigen × vaccinia antiserum.
B. Ectromelia antigen × vaccinia antiserum.
C. C.P.D. antigen × C.P.D. antiserum.

Fig. 3b. Diffusion patterns showing cross reactions between C.P.D. and vaccinia.

A. C.P.D. antigen × C.P.D. antiserum.
B. Vaccinia antigen × C.P.D. antiserum.
C. C.P.D. and vaccinia antigen × C.P.D. antiserum.
D. Vaccinia antigen × vaccinia antiserum.

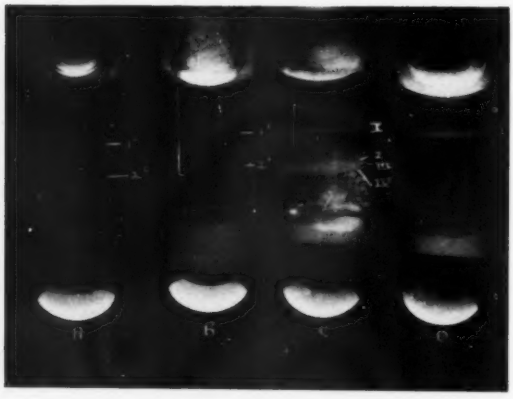

Fig. 3c. The effect of heat on C.P.D. and vaccinia antigen diffusion patterns.

A. C.P.D. antigen × C.P.D. antiserum.
B. Heated C.P.D. antigen × C.P.D. antiserum.
C. Vaccinia antigen × vaccinia antiserum.
D. Heated vaccinia antigen × vaccinia antiserum.

TABLE 3.

Cross neutralisation between C.P.D., ectromelia and vaccinia.

Virus	Test animal	Normal serum titre	Antisera					
			C.P.D.		Ectromelia		Vaccinia	
			Titre	N. 1	Titre	N. 1	Titre	N. 1
C.P.D.	Sheep (2)	4·7	2·7	2·0	4·7	0	4·7	0
	Rabbits (6)	4·7	3·6	1·1	3·7	1·0	4·0	0·7
	All results pooled	4·7	3·1	1·6	4·0	0·7	4·3	0·4
Ectromelia	Mice Exp. 1	6·8	5·0	1·8	6·0	0·8	4·5	2·3
	Mice Exp. 2	7·8	6·8	1·0	6·0	1·8	6·5	1·3
	Mean	7·3	5·9	1·4	6·0	1·3	5·5	1·8
Vaccinia	C.A. Exp. 1	6·2	5·9	0·3	5·2	1·0	4·2	2·0
	C.A. Exp. 2	6·6	6·3	0·3	5·7	0·9	5·1	1·5
	Mean	6·4	6·1	0·3	5·45	0·95	4·65	1·75

Cross neutralisation tests.

The results of the cross neutralisation tests (Table 3) show no significant cross neutralisation between vaccinia and C.P.D., but C.P.D. antiserum gave substantial neutralisation against ectromelia virus. The reverse did not hold, but the ectromelia antiserum was very weak in neutralising activity against the homologous virus, and it is therefore not surprising that it failed to show activity against a heterologous virus. The significant cross neutralisation between vaccinia and ectromelia viruses and antisera were to be expected for these viruses have been shown to be closely related immunologically.

DISCUSSION.

The present investigation shows that the C.P.D. virus has a soluble complement fixing antigen and a soluble precipitin. The soluble complement fixing antigen is heat stable and is of small particle size for it is not sedimented at 40,000 r.p.m. (140,000*g*) for 1 hour in the model "L" spinco ultracentrifuge. The precipitin appears to consist of two fractions, but as Gispen (1955) points out the fractions would have to be identified by absorption methods for they could be similar haptenes of different molecule size.

The cross complement fixation tests and double diffusion tests both show an immunological relationship between C.P.D., vaccinia and ectromelia. While the cross neutralisation tests show a definite relation between C.P.D., and ectromelia, but no significant relationship between C.P.D. and vaccinia.

The C.P.D. virus is therefore similar to the mammalian pox group in possessing a heat stable complement fixing antigen which will cross react with vaccinia

and ectromelia; it is also similar in that it possesses soluble precipitins one of which corresponds with zone III of vaccinia and ectromelia.

These immunological relationships of the C.P.D. virus together with its similarity in size, shape and disease manifestation to the mammalian pox group, constitute a substantial relationship with the group. On these grounds the writer is of the opinion that this virus should be included in that group.

SUMMARY.

C.P.D. virus was compared with vaccinia, ectromelia and fowl pox viruses by cross complement fixation tests, double diffusion tests and cross neutralisation tests. The results show immunological cross reactions between C.P.D., vaccinia and ectromelia viruses, and on these grounds it is suggested that the C.P.D. virus should be included in the pox group.

Acknowledgments. I wish to express my appreciation of the help and encouragement I have received from Professor J. A. R. Miles, especially for carrying out the cross neutralisation tests and the reading of the manuscript.

I also wish to thank the Department of Scientific and Industrial Research for the preparation of the electron micrograph.

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CORTISONE AND ANAPHYLAXIS[1]

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Cortisone is known to influence the allergic reaction profoundly. The mechanism of action is thought to relate largely to suppression of antibody production thereby gradually reducing the allergic state since antibody is no longer available to attach to cells of the shock organ (Germuth, Oyama and Ottinger, 1951). These workers also reported that the systemic passive Arthus reaction was not inhibited. Peripheral effects in man relating to inhibition of the action of histamine and other H substances are not regarded as significant.

It has been shown previously (Trethewie, 1951a, b, 1952) that salicylates inhibit the release of histamine and S.R.S. in anaphylaxis. In adequately conducted trials (M.R.C., 1954, 1955; Bayliss, 1955) cortisone showed no superiority clinically over aspirin in the treatment of early acute rheumatoid arthritis, although the former reduced the sedimentation rate more and haemoglobin levels were slightly higher.

This report concerns the effect of cortisone on the release of histamine and S.R.S. in anaphylaxis and a quantitative analysis has been made of the effect of cortisone on the peripheral responses to histamine and S.R.S. Clinically, in the past, cortisone preparations were not able to be injected intravenously rapidly enough to give high blood levels and this has probably accounted for the lack of evidence of significant reduction of the peripheral response to histamine in man.

Method.

Isolated intestine.

Tests for histamine and S.R.S. were carried out on the isolated jejunum of the guinea-pig suspended in Tyrode. Histamine was estimated as histamine acid phosphate. It has been shown previously by Bartosch, Feldberg and Nagel (1932) that the substance causing immediate contraction of the gut which is liberated from the anaphylactic lung is histamine and this has been further confirmed with antihistamine studies (Trethewie, 1951b). When cortisone was tested it was added as cortisone acetate (Evans) in suspending agent in a concentration of 1 in 70,000-1 in 140,000 and left in contact with the gut for periods of ten up to sixty-eight seconds. S.R.S., for these latter experiments, was obtained from guinea-pig lung, which also contains histamine. In some experiments, soluble hydrocortisone was used.

[1] Technical assistance for this work was aided by a grant from the National Health and Medical Research Council.

Perfusion experiments.

Guinea-pigs weighing 80-100 gm. were sensitized by the subcutaneous injection of 15 mg. of crystalline egg albumin dissolved in 0·5 ml. Tyrode. When the animals weighed 300-510 gm. three to five months later, the animals were stunned by a light blow on the head and the isolated lungs perfused with Tyrode *via* the pulmonary artery in an incubator at 37° C. Antigen (20 mg.) was injected into the pulmonary artery, the flow interrupted for 1½ min., and the flow then re-established. For the cortisone experiments Evans' B.P. cortisone acetate 5 p.c. in suspending agent was used. This enabled perfusion to proceed for a reasonable time while the cortisone, added to 600-700 ml. Tyrode in a concentration of 1 in 4,000 or 1 in 10,000, remained in suspension. The delivery flask was agitated regularly.

EXPERIMENTAL.

The influence of cortisone on the response of the gut to histamine and S.R.S.

First it was necessary to determine the concentration at which cortisone causes depression of the response of the gut to histamine and S.R.S. since these substances were assayed in the perfusate obtained from the anaphylactic lung.

It was found that when cortisone was left in contact with the gut in small dosage for a few seconds there was little reduction in the response to histamine. For example, in one instance 0·1 mg. cortisone (= 1 in 70,000 dilution in the bath) added to 0·1 μg. histamine left in contact with the gut for ten seconds produced slight depression only of the response as compared with that to 0·1 μg. histamine without cortisone. 0·05 mg. cortisone added to 0·1 μg. histamine produced no demonstrable reduction in the response to histamine when left in contact for ten seconds while 0·2 mg. produced a reduction of about 15 p.c. (H_c, Fig. 1). On the other hand, when cortisone was left in contact with the gut for 60 seconds the reduction in response was much greater. 1 mg. cortisone added to the bath (1 in 7,000) and left in contact for 1 minute caused practically complete inhibition of the response to 0·1 μg. histamine, and recovery, after washing out the bath several times, was not complete for 12 minutes (C_1, Fig. 1). When 0·1 mg. cortisone was added to the bath (1 in 70,000) and left in contact for 1 minute the response to 0·1 μg. histamine was reduced by approximately 60 p.c. ($C_{0.1}$, Fig. 1), and 0·05 mg. cortisone (1 in 140,000) reduced the response of the gut by 40 p.c. when left in contact for one minute ($C_{0.5}$, Fig. 1). Contact for longer periods of four up to eight minutes caused greater reduction (50-75 p.c.) in response though, of course, the gut could not be supplied with fresh Tyrode during this time. When cortisone was added to the Tyrode delivery flask regular washing-out was possible. In this experiment adsorption of cortisone acetate can occur on to the tubing. Occasionally the first histamine response obtained in the presence of cortisone was increased. When the Tyrode delivery flask contained 1 in 100,000 and 1 in 140,000 cortisone the histamine response diminished progressively to 40 p.c. and 50 p.c. respectively, the response then remaining constant for twenty minutes.

It is evident, therefore, that it is quite possible to assay histamine outputs from the perfused lung in the presence of cortisone provided the concentration does not exceed 1 in 70,000 and the sample is not left in contact for more than 10 seconds. Employing this procedure, perfusate from the perfused anaphylactic lung injected with egg albumin has been shown to contain large amounts of histamine.

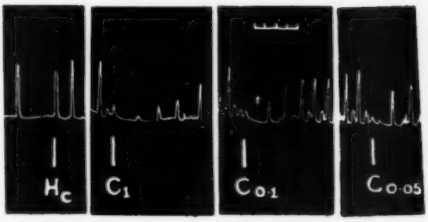

Fig. 1. Responses of the isolated jejunum of the guinea-pig to histamine with and without added cortisone. The unlettered responses are to 0·1 μg. histamine. At H_c response to 0·1 μg. histamine to which 0·2 mg. cortisone was added: left in contact 10 seconds, contraction completed at 8 seconds. At C_1 1 mg. cortisone added to the bath left in contact 60 seconds when first histamine response tested and bath washed out. At $C_{0.1}$, 0·1 mg. cortisone left in contact 60 seconds similarly. At $C_{0.05}$, 0·05 mg. cortisone added to the bath, left in contact 60 seconds similarly. The unlettered responses are to 0·1 μg. histamine. Time in minutes. Further details in text.

The response to S.R.S. is also reduced dramatically in the presence of cortisone. The findings in one experiment are illustrated in Fig. 2, where at S is shown the response to 40 mg. unboiled guinea-pig lung. The immediate contraction is due to histamine and the considerable appended delayed relaxation is the latter part of the S.R.S. response. The second response to 40 mg. unboiled lung is even greater. Following the addition of 1 mg. cortisone to the bath (1:7,000) acting for 68 seconds the response to 40 mg. lung (at C_1S) is almost completely abolished. At $C_{0.1}S$ the response to 40 mg. lung following the injection of 0·1 mg. cortisone (1:70,000) acting for 68 seconds was greatly reduced. The histamine response is still of some magnitude (not much less than previously, which was in fact a response to a supra-maximal dose) while the S.R.S. response was almost abolished, relaxation occurring largely immediately the gut bath was washed out.

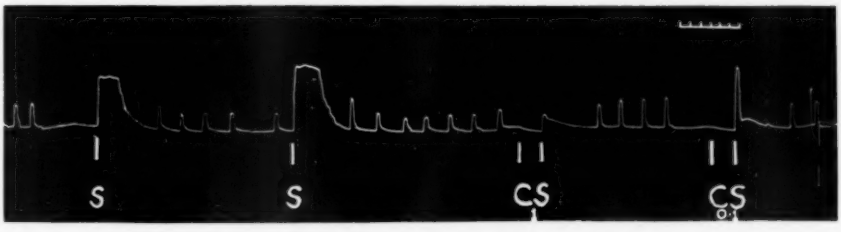

Fig. 2. Responses of the isolated jejunum of the guinea-pig to S.R.S. and cortisone. At S, 40 mg. unboiled lung extract added to the bath and left in contact 15 seconds. At C_1, 1 mg. cortisone added to the bath left in contact 68 seconds. At $C_{0.1}$, 0·1 mg. cortisone added to the bath, left in contact 68 seconds. The unlettered responses are to 0·1 μg. histamine. Time in minutes. Further details in text.

TABLE 1.

Output of histamine in perfusate.

Exp. No.	Cortisone conc.	Concentration H(μg./ml.) in perfusate Time after injection antigen		Lung H μg./g.	Total histamine output (μg.)
		0-5 min.	5-15 min.		
1	—	0·05	0·03	27	0·8
2	—	0·15	0·03	20	1·9
3	—	0·10	0·08	9	0·9
4	1/10,000	0·67	0·29	15	7·9
5	1/10,000	0·08	0·03	16	0·9
6	1/4,000	0·40	0·13	19	2·7
7*	—	—	—	27	0·0

* 10 mg. cortisone injected into the pulmonary artery of an anaphylactic lung, the sample times now being 0-7 and 7-15 minutes, (no antigen injected).

Liberation of histamine and S.R.S. in anaphylaxis in the presence of cortisone. Control experiments.

Histamine and S.R.S. are readily detected in the perfusate obtained from the perfused anaphylactic lung following the injection of antigen, and the concentration of histamine in perfusate in three such experiments (Exp. 1, 2, 3) is shown in Table 1.

Cortisone perfusions.

When cortisone was added to the perfusing fluid in a concentration of 1 in 10,000, histamine was likewise very readily demonstrated in perfusate following the injection of antigen. For example, in Exp. 4 the lungs of a guinea-pig weighing 360 gm. were perfused with Tyrode, to which cortisone had been added (1 in 10,000; 70 mg. in 700 ml.). After the blood was perfused out of the lung the rate of perfusion was slowed to 1 ml. per minute, and subsequently 3·8 ml. of fluid were collected over 4 minutes. Assay on the isolated gut of a guinea-pig failed to detect the presence of any histamine or S.R.S. Following the injection of 20 mg. egg albumin perfusate collected for the first five minutes contained histamine 1 in 1·5 millions and a small amount of S.R.S. In the succeeding 10 minutes the histamine concentration was 1 in 3·5 millions, and a considerable quantity of S.R.S. was also present. The lung weighed 4·55 gm. after perfusion and contained 15 μg. histamine per gm. These findings are recorded in Table 1 (Exp. 4). In another experiment the histamine concentrations were 1 in 12 millions and 1 in 30 millions, 0-5 and 5-15 minutes respectively after injection of antigen and a considerable quantity of S.R.S. was detected in the perfusate (Exp. 5, Table 1). In another experiment the concentration of cortisone was increased to 1 in 4,000 and again a considerable output of histamine was detected (Exp. 6, Table 1).

The output of histamine in the three cortisone experiments described was

three times as great as that in the control experiments. While this is not significant statistically, it was thought worth while to determine if a large dose of cortisone could cause the release of histamine although the lung did not release histamine into the perfusate when perfused with cortisone (1 in 10,000) prior to the injection of antigen. It is to be remembered that there is evidence that antihistamine liberates histamine on injection. In Exp. 7, 10 mg. cortisone were injected into the pulmonary artery of a perfused anaphylactic lung. The flow was interrupted for 1½ minutes and was then re-established. Perfusate collected during the succeeding 0-3, 3-7 and 7-15 minutes was free of histamine and S.R.S. The lung contained 27 μg. histamine per gm. after perfusion and weighed 4·72 gm.

DISCUSSION.

It is clear from the above experiments that the depressant effect of cortisone on the anaphylactic reaction cannot be due to suppression of release of pharmacologically active substances unless the Tyrode perfused organ reacts differently from the blood-filled organ *in vivo*. It has also been shown that the release of histamine from blood in haemolytic reactions *in vitro* is not inhibited by cortisone (Carryer and Code, 1950). Previous work had suggested that cortisone and A.C.T.H. acting through cortisone depressed the sensitivity of the tissue injury response, thereby inhibiting the release of histamine and other substances, e.g. Ungar (1944) presented evidence to indicate such inhibition occurred regarding the release of histamine by peptone.

The occasional clinical finding of an accentuation of symptoms in status asthmaticus following initiation of intravenous cortisone therapy might be explained, not so much by unexpected "sensitivity" of the subject to cortisone as to some altered response to the released pharmacological substances. The increased histamine output in the experiments recorded in this paper suggested a possible explanation, but, though large, the difference in output in the presence of cortisone is not significant statistically. Further cortisone was not found to liberate histamine on injection. This was not a remote possibility, since it is known that antihistamine can liberate histamine on intravenous injection. Of course, *pari passu* with this release with large doses, the effector organ ending is blocked, as is shown here, so that the released histamine does not produce pharmacological effects. If, however, an increased histamine output occurred, say, with cortisone, and for some reason the peripheral depressant action of cortisone was not produced so soon or to an equal degree in a patient receiving intravenous cortisone, a possible flare-up of symptoms might occur. Alternatively, the peripheral end organ might pass through a short supernormal phase of excitability before depression develops, and this was occasionally observed with the gut in the dosage we employed, but it was very evanescent. Harmful effects might also be produced by action of cortisone on heart muscle cells causing polymerisation of actin causing relaxation and asystole.

It is of interest to note that antihistamines, except possibly phenyl-methyl-tetrahydroazofluorene, do not usually inhibit the acid secretory response of the stomach to histamine. It may be that the blocking effect of antihistamine is not very marked and the increased output of histamine compensates for any slight blocking effect and so acid is secreted. Spiegelhoff (1953) showed that A.C.T.H. and cortisone increased daily rhythmical secretion of gastric acid in man in therapeutic dosage provided the pituitary and suprarenal gland were healthy, but not when they were diseased. In one experiment on the decerebrate cat we found that the output of free HCl following histamine injection was largely abolished by cortisone.

Cortisone reduces the amount of circulating antibody by inhibiting antibody production (Germuth, Oyama and Ottinger, 1951) and in larger dosage can prevent completely the production of antibody to a sensitizing agent (Bierich and Bierich, 1953). This effect probably accounts for the improvement in patients suffering from allergic conditions following moderate dosage of cortisone over a period of days.

The Arthus reaction in the skin of rabbits has been considered as not reduced in the presence of cortisone (Germuth, Oyama and Ottinger, 1951), but two hours after challenge the reaction to antigen was smaller and it was not till later—4, 6 and 24 hours—that the reactions were of equal intensity. This might have been due to excretion of the cortisone so that its concentration fell to sub-effective levels. Tillotson (1951) described great reduction of the Arthus reaction by cortisone due to modification of antibody. Hughes, Lecompte and van Gauenberge (1954) reported inhibition of the anaphylactic mesenteric response in rabbits following cortisone injected for two days before and on the day of the experiment. They commented that cortisone increases vascular tone and limits permeability of vessels.

It is difficult to relate the gross reduction of the response of the gut to histamine in the presence of cortisone, as shown in this paper, with the finding that patients treated with A.C.T.H. and cortisone do not show reduced reaction to antigen or histamine (Feinberg, Dannenberg and Malkiel, 1951) unless this is due to difference in dosage. The dosage of cortisone and A.C.T.H. used in four of these sixteen subjects can be inferred from their Table 1. In these patients the total dose of A.C.T.H. was 390, 500, and 500 mg. and of cortisone (in two cases) 3,000 mg. and 1,150 mg. One patient received both cortisone and A.C.T.H. These drugs were given over 8, 15, 7; and 28 and 8 days respectively. The daily dose presumably therefore was about 30-70 mg. A.C.T.H. and about 110-150 mg. cortisone. We found cortisone 1/140,000, acting for only one minute, produced significant reduction in the response of the gut to histamine. Then at any one time there would need to be a quantity of approximately 35 mg. in the blood in man to reach this concentration. The dosage referred to above would not reach this level. Nevertheless, not infrequently it is required to give 100 mg. or even 200 mg. cortisone slowly by continuous intra-

venous drip during one or two hours in order to reduce status asthmaticus. Soluble hydrocortisone sodium succinate is now available for more rapid injection. Such therapy could achieve a level of 35 mg. in the total blood volume at one time. Cooke *et al.* (1951) also reported that the response of the skin to histamine is not abolished by A.C.T.H. and cortisone therapy. Here, also, the explanation may lie in the possibility that the dosage, while being adequate to inhibit antibody production, is below that necessary to produce inhibition of the peripheral response. Williams, Heiple, and Ebert (1954) in investigations in the rabbit found that the threshold of response to histamine was elevated or unchanged following daily doses of cortisone (5 mg.), the test being carried out on the fourth day after three daily injections. Chappell, Ebert and Barclay (1952) have shown that cortisone inhibits the cutaneous histamine response in rabbits as measured by diffusion of Evans' Blue dye. We have found (Trethewie, 1957b) that 25 mg. soluble hydrocortisone injected intravenously inhibits the skin weal response of the rabbit to submaximal doses of histamine. Kind (1954) showed that cortisone inhibited the temperature drop produced by histamine in mice, but suggested this effect might have been due to a potentiation of vasoconstriction by endogenous adrenalin. Durwood Smith (1951) found that cortisone accelerates recovery of the smooth muscle of vessels following vasoconstriction due to histamine and the effect may be on amino-oxidase or other enzymes accelerating recovery. This is in contradistinction to the effect of cortisone on smooth muscle in gut and bronchi. We are pursuing these studies in man.

Diagrammatic representation of site of action of cortisone in anaphylaxis.

It has been reported earlier (Trethewie, 1951a, b) that salicylates inhibit the antigen-antibody reaction in a concentration between 25 and 50 mg./100 ml. which corresponds to the effective therapeutic blood level in rheumatic fever, generally regarded as 35 mg./100 ml. The mechanism of action of salicylate in the anaphylactic response is shown diagrammatically in Fig. 3. The site of salicylate block is indicated and as a consequence no histamine or S.R.S. is released (Trethewie, 1952). Salicylate has no effect, in therapeutic dosage, on the

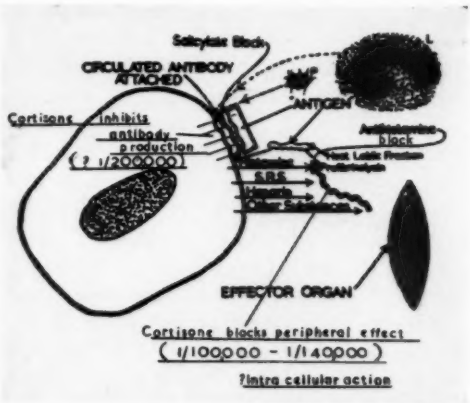

Fig. 3. Scheme of the anaphylactic response showing: attached antibody, sensitized cell, union of sensitizing antigen, relation of platelet (P) and leucocyte (L) to response, rôle of profibrinolysin and heat labile fraction, release of H-substances and effector organ. The site of antihistamine block, salicylate block and cortisone action on antibody and periphery is indicated. Further details in text.

peripheral response to histamine or S.R.S. Similar findings were obtained with colchicum, aminophyllin (Trethewie, 1955a), and B.T.Z. (Trethewie, 1955b). The findings with cortisone are in sharp contrast. Here there is no inhibition of the antigen-antibody reaction, but in a concentration of 1/100,000-1/140,000 there is a gross reduction in the response to histamine (as is similarly obtained with antihistamine) and the response to S.R.S. is likewise also depressed. These findings are indicated in Fig. 3. In smaller concentration cortisone impairs antibody production so that after a period the allergic response is repressed by cortisone since insufficient antibody is produced and therefore sensitization of cells by attachment to antibody can no longer occur (Fig. 3). Such a representation clarifies the apparent conflict in the different results described by various workers, e.g., concerning the Arthus reaction, especially when it is remembered that reduction of antibody production by lower concentrations of cortisone can only be effective when the initial antibody level has fallen over a period of days. The inhibition of the peripheral response by a large dose takes place rapidly and may be due to an intracellular action. Cortisone stimulates polymerisation of actin (Zador, 1957) and this may be the intracellular mechanism causing relaxation of smooth muscle, of, say, bronchi and gut, and depressing their response to histamine.

Comparative effect of salicylate and cortisone.

It has been averred previously (Trethewie, 1951a, b, 1954a, 1955a) that the value of salicylate in such conditions as rheumatic fever, asthma and acute rheumatoid arthritis is very likely related to this suppression of the antigen-antibody reaction with consequent inhibition of release of histamine, S.R.S. and other H substances. Further, since this effect occurred with the isolated organ in the absence of the pituitary and the suprarenal gland a mechanism involving cortisone or A.C.T.H. was regarded as superfluous although Keleman, Majoras, Ivanyi and Kovacs (1950) had indicated that salicylates owed their effect on the allergic reaction to the release of A.C.T.H. and cortisone. That this suggestion is unlikely has been further confirmed by Bayliss and Steinbeck (1954) and Smith, Gray and Lunnon (1954) who showed that there is no increase in the level of adrenal steroids in the blood or in the urine following salicylate therapy. In rheumatic fever, unfortunately, other effects of cortisone may increase the inflammation so that it is usually contra-indicated except in acute heart failure. It is known that salicylate and cortisone show no superiority of one over the other in producing amelioration of the symptons in early acute rheumatoid arthritis (M.R.C., 1954, 1955; Bayliss, 1955) and the nett result of each pharmacologically is to reduce the impact of the allergic response in the ways described above. It suggests that allergy probably plays no unimportant part in the production of symptoms in early acute rheumatoid arthritis, a view held by many. It is also worthy of mention that Ungar, Damgaard and Hummel (1952) have demonstrated that salicylate inhibits fibrinolysin activity and this is especially worthy of note since Ungar and Mist (1949) relate fibrinolysin activity to the allergic reaction as is illustrated in Fig. 3.

SUMMARY.

Cortisone, in a concentration of 1/4,000-1/10,000, has no inhibitory effect on the release of histamine or S.R.S. in anaphylaxis.

Cortisone, in a concentration of 1/7,000-1/140,000, causes considerable depression of the response of the gut to histamine and S.R.S.

The significance of the above findings is discussed, especially in relation to the pharmacology of salicylate and responses in early acute rheumatoid arthritis.

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APPARATUS FOR VACUUM DISTILLATION AND FOR "FREEZE-DRYING"

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This paper described three simple, relatively inexpensive and easily made pieces of apparatus for the use of biochemists who wish to remove water from such fluids as dialysates and chromatographic eluates and of micro-biologists who have to "freeze-dry" cultures of living organisms.

The first is a compact metal "shell" condenser the emergent cooling water from which operates a water vacuum pump which also removes the distillate. The materials used were as follows: a piece of 2 inch copper water pipe 11 inches long, 4 pieces of copper tube each 0·375 inch outside diameter and 9 inches long, four round discs of brass 0·06 inches thick for the ends of the jacket and the heads of the condenser, short pieces of copper tube of 0·5 inch and 0·31 inch external diameter for the connections and a short piece of round brass rod 1·5 inches diameter from which was turned the vacuum connection to the still. Jewellers' "silver solder" was used to solder the joints. The vacuum connection was tapered to fit a standard rubber stopper. Before the ends were soldered in place the central portion was boiled in water and then tested to ensure that there were no leaks. The openings may be placed round the circumference of the outer case to suit local requirements. The water-pump is placed with its side tube level with or below the outlet tube for the distillate, which tube is near the bottom of the condensing chamber. A test was made with a 2 l. distilling flask having a short exit tube of 16 mm. bore; when heated in a water-bath maintained at 86° C. the temperature of the liquid in the flask did not exceed 27° C., about 9° above that of the cooling water. In 45 minutes 1 litre of water was distilled.

The second apparatus is a simple "freeze-drier" for drying cultures of micro-organisms in which calcium carbide is used to trap the water-vapour. It is a development from previous work (Holden and Scott, 1950; Holden, 1954) and has proved so satisfactory and cheap to use that in this institute the cultures are now normally dried in it. The body of the trap was made of iron with "silver-soldered" joints. The inner carbide holder has a cone to fit the socket

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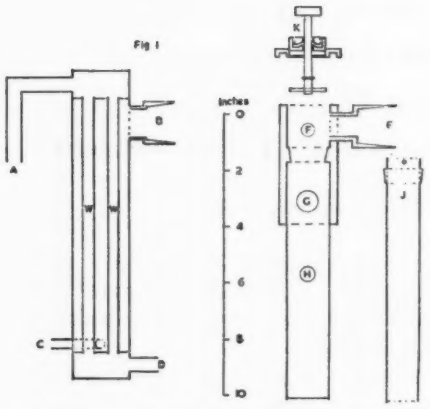

Fig. 1. Water-pump condenser. A, outlet for cooling water to water-pump; B, inlet for water-vapour from the still; C, outlet for condensed water to the side tube of the water-pump; D, inlet for cooling water from the water main; W.W., condenser tubes (two only shown). Carbide trap for small "freeze-drier". E, inlet for water-vapour from the manifold; F, connection to Pirani gauge; G, outlet to vacuum pumps; H, air admittance valve; J, carbide holder with cone to fit the body of the trap; K, lid with handle to lift carbide holder.

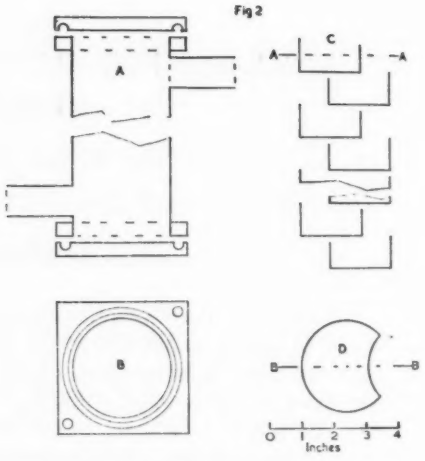

Fig. 2. Large carbide trap for "freeze-drier". A, vertical section of outer case; inlet near the top; B, end plate to show positions of studs and the groove for the gasket; C, vertical section of carbide holder along B-B; D, horizontal section of carbide holder along A-A.

in the body so that during drying the water-vapour passes through the holder. It can be raised with the handle passing through the Wilson seal in the lid. The holder is closed at the bottom with a disc of fine copper gauze on which rests a loose pad of cotton wool. The glass manifold resembles in shape the one described (Holden, 1954), but it now holds 21 ampoules similar to those used for drying sera. The manifold can be rotated about a horizontal axis so that the ampoules are in a freezing bath at $-10°$ C. to $-12°$ C. during drying and are sealed in a horizontal position to avoid heating their rubber connections to the manifold. A small two-stage rotary pump with a speed of about 20 l. per minute is used with an oil condensation pump for producing a higher vacuum before sealing off the ampoules. There are no valves within the system as there is no "rapid cycling". The contents of the ampoules are frozen before drying in "dry ice", a "deep-freeze" or with a calcium chloride-ice bath. The plugs are not removed at any stage nor is air or other gas admitted to the ampoules, thus avoiding two risks of contamination. Before sealing off the ampoules the carbide container is raised to provide a clear passage and the pressure is reduced for

half an hour to less than 1 micron, the limit of the Pirani gauge used. In making the gauge head it was found essential to "silver-solder" the tungsten filament to a fine molybdenum wire and to cover the joint with shellac, thus protecting it from the attack by acetylene which spoilt a gauge head in which the filament was clamped as in an electric light bulb. After using the drier the carbide is screened through "fly-wire", 14 wires to the inch; the portion that does not pass is kept in an air-tight jar and put at the top of the container when the drier is next used; thus little carbide is wasted. To ensure thorough drying the ampoules are sealed off 24 hours after drying is started, during the last 16 of which they are kept at room temperature.

The third apparatus is a larger "freeze-drier" suitable for evaporating up to 150 ml. of water. To accommodate a larger quantity of calcium carbide without risk of blocking the passage of air a container of a special form was made. It consists of a tube of thin sheet-iron sliding easily within the outer cylindrical case of the trap. By cutting a series of slots, bending back portions of the wall of the tube as shown and soldering in place the flat discs of iron an arrangement was produced equivalent to a stack of fourteen trays each 0·8 inch deep with a gap above each 0·2 inch by 1·9 inch wide for the gases to flow from one tray to the next below. The final design and manufacture of this carbide container are due to Mr. K. Lynikas of this institute. The outer case of the trap is of steel tube 18 inches long and 3 inches diameter. Its ends are closed with iron plates each having a 0·25 inch round rubber gasket in a retaining groove. Each plate is held to the body of the trap with two five-sixteenth inch studs and nuts. The two-stage rotary pump used has a nominal speed of 58 l. per minute.

In use the temperature of the trap rises to about 60° C. A larger one would be water-cooled. The acetylene produced is sent to the outer air through a fume cupboard. Below the inner carbide container is a small filter. A vacuum switch is added to stop the motor at once in the event of an ampoule breaking; the pressure in the inlet to the carbide trap is read with a Pirani gauge. During the early stages of drying it stands at about 1 mm., gradually falling to less than 100 microns. About 80 ml. of water can be evaporated during the day and in 24 hours 150 ml. were evaporated without the carbide having to be replenished. After use the inner carbide container is covered with a piece of four inch galvanized iron pipe with a wire screen on one end. When inverted and shaken gently the slaked lime is readily separated from the remaining carbide which can be re-used.

SUMMARY.

Brief descriptions are given of: a laboratory condenser of which the outflowing cooling water operates the water-pump which evacuates the still and which

removes the distillate; a small "freeze-drier" for cultures of micro-organisms using CaC_2 to absorb the water; and a larger "freeze-drier", also using CaC_2.

REFERENCES.

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